THE ALKALOIDS OF *STRYCHNOS LUCIDA*

by F. H. SHAW AND I. S. DE LA LANDE[1]

(From the Department of Physiology, University of Melbourne).

(Accepted for publication, 9th January, 1948.)

Strychnos lucida is an Australian *Strychnos* species which botanically is closely related to *Strychnos nux vomica*. *Strychnos lucida* has not been examined in detail for alkaloids, although it has been assayed for the strychnine content. Watson (1940–41) assayed the seed by a method based on the methods of the British Pharmacopoeia (1932) and the United States Pharmacopoeia (X) assay of *S. nux vomica* seed, and reported 0·84 p.c. strychnine and 1·55 p.c. brucine. The New South Wales Poison Plants Committee (1942) records a strychnine content of 0·57 p.c. strychnine. However, the part of the plant assayed and the assay method are not recorded.

In the present investigation, the presence of strychnine and brucine in the seed has been confirmed by their isolation, and their probable presence in the bark and leaf indicated by assay data. In addition, non-strychnine-brucine alkaloid fractions have been isolated from the seed and leaf. These have been tentatively called lucidine-S and lucidine-L respectively. Under the conditions of the B.P. (1932) or U.S.P. XI (1936) methods for the determination of strychnine, these fractions are estimated as strychnine, and the actual strychnine content must therefore be lower than indicated by these methods. This has been further shown by the following: the amount of strychnine isolated from a large sample of seed corresponded roughly to a 0·1 p.c. yield whereas the U.S.P. assay had indicated more than 1 p.c. of strychnine; experiments on some of the supposed strychnine residues from U.S.P. assays of the seed and leaf showed that these were approximately one-fifth as toxic as strychnine in mice.

Lucidine-S and lucidine-L are amorphous, and have similar solubilities to brucine. Both were isolated in small quantities from the plant by the same method, and appear to be identical. However, neither has been characterized owing to the inability to isolate the bases or their salts in a crystalline condition, and hence it is not known whether these alkaloid fractions are homogeneous or not.

Assay of S. lucida.

The U.S.P. assay method for the determination of strychnine in *S. nux vomica* was used in the present investigation. In individual cases, the following modifications were applied:

[1] This work was carried out with the aid of a grant from the National Health and Medical Research Council.

(1) The alkaloids were completely extracted from 10 gm. of the plant with chloroform after preliminary treatment with lime according to the method of Shaw and Trautner (1945).

(2) The total alkaloid was estimated by weighing the alkaloid residue prior to redissolving in N/3 sulphuric acid, oxidizing to remove the brucine.

(3) The alkaloid residue remaining after oxidative removal of brucine, which in *Strychnos nux vomica* consists mainly of strychnine, was estimated gravimetrically. This alkaloid residue (called a.r. for the remainder of the paper) in the U.S.P. assay method is not weighed but dissolved in dilute sulphuric acid and estimated volumetrically by back titration with sodium hydroxide.

(4) The alkaloid residue (a.r.) was first estimated gravimetrically, and then redissolved in dilute sulphuric acid and estimated volumetrically.

(5) The toxicity (approximately L.D.50) of a.r. in mice was estimated.

TABLE 1.

No.	Sample.	Total alkaloid (p.c.).	Brucine* (p.c.).	Non-brucine alkaloid (p.c.).		Strychnine estimated from the toxicity of a.r. (p.c.).	Remarks (modifications employed).
				Gravimetrically (a.r.).	Volumetrically.		
1	Seed	3·7	2·2	1·5		0·3	(2), (3)
2	Seed	4·5	2·4	2·1	1·7	0·4	(2), (4), (5)
3	Seed	3·4	1·5	1·9			(2), (3)
4	Seed	2·0	1·5	0·5			(2), (3)
5	Seed	3·2	1·8	1·4	0·7	0·3	(2), (4), (5)
6	Seed	4·0	2·2	1·8			(1), (2), (3)
7	Leaf	1·2	0·8	0·4			(1), (2), (5)
8	Leaf	2·2	1·5	0·7			(1), (2), (5)
9	Leaf	2·0	1·2	0·8	0·3	0·2	(2), (4), (5)
10	Bark	5·9	4·8	1·1		0·2	(1), (2), (5)
11	Husks and pulp of fruit	0·24	0·23	0·01			(2), (3)

* p.c. Brucine = p.c. Total alkaloid — p.c. a.r.

These results indicate that, in the seed, the percentages of total alkaloid and non-brucine alkaloid are subject to considerable variation.

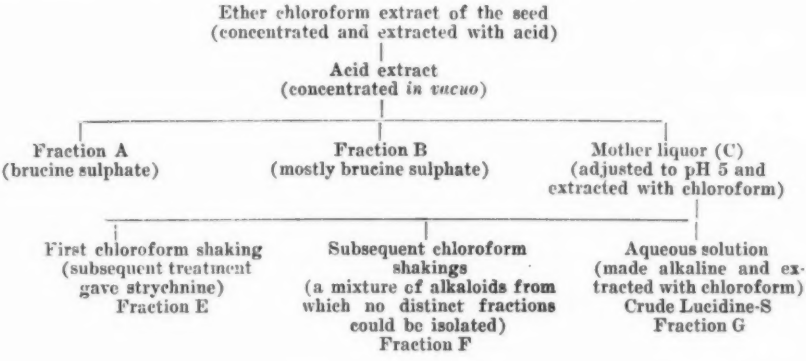
There is a very marked discrepancy between the percentage of strychnine as determined by volumetric, gravimetric, and toxicity determinations. Jensen (1916) in an examination of the accuracy of the gravimetric (Method (3)) and the volumetric estimations of strychnine in *S. nux vomica* seed concluded that the volumetric estimation was more accurate provided that the result was corrected for the presence of a small amount of strychnine citrate. If this correction is not made, on his data, the gravimetric method is more accurate.

Methods of determining the strychnine content of *S. nux vomica* seed from the toxicity of the alkaloid residue (a.r.) (Method (5)) are not reported in the literature, but their importance in this case is evident.

The very marked discrepancies obtained by us between the three methods suggest that the accuracy of the estimation of the strychnine content of *S. lucida* by any of these methods is doubtful. The toxicity results suggest that the alkaloid residue (a.r.) which in *S. nux vomica* is assumed to consist mostly of strychnine, consists of either a high proportion of strychnine associated with an actively antagonistic substance, or a low proportion of strychnine associated with physiologically inert material, presumably alkaloidal. The latter alternative is suggested by the isolation of a small amount of a physiologically inert alkaloid fraction (lucidine-S) from the seed and leaf. As this is not appreciably removed by oxidation under the conditions for removal of brucine, this is present with strychnine in the alkaloid residue (a.r.), and hence the strychnine content, estimated volumetrically or gravimetrically, is high. It is likely that similar considerations apply to the B.P. assay method which is fundamentally similar to the U.S.P. method.

Isolation of the Alkaloids.

The following scheme will assist in an understanding of the various steps taken during the isolation of the various fractions from the seed.



EXPERIMENTAL.

1·3 kg. of the seed and husk of a mixture of samples Nos. 1, 2 and 3 were treated with 50 ml. of N alcoholic sulphuric acid thoroughly moistened with alcohol, and allowed to dry for several hours. The mixture was then extracted for several hours with a mixture of ether-petroleum ether (1:1) for about three hours. The seed was then dried, thoroughly stirred with 200 gm. of calcium hydroxide and water until uniformly mixed throughout, and allowed to dry overnight. The mixture was then extracted with ether chloroform (2:1) until the bulk

of the alkaloid had been extracted. The ether-chloroform extract was then concentrated to a thick syrup and the alkaloid completely extracted with N/5 aqueous sulphuric acid. The acid extract was brought close to neutrality with ammonia, and concentrated *in vacuo* at 35–40° C. Two crystalline fractions (A and B) were removed before oily material began to separate out from the aqueous concentrate. Sufficient water was added to redissolve this oily material (aqueous extract = C).

Fractions A and B were shown to be predominantly brucine sulphate (solubility = 1:70). A small portion of fraction A was redissolved in hot water, and the base precipitated by rendering the solution alkaline with sodium hydroxide. The precipitated base (A) was redissolved in the minimum volume of methanol, and reprecipitated by the addition of water. The solution was filtered, and the crystalline base dried *in vacuo* at 70° C. The base melted at 175° C. and the M.P. was not altered by further recrystallization from hot water (cf. brucine 178° C.). With dilute nitric acid, it gave the intense red coloration characteristic of brucine.

In an experiment, 0·4 gm. of the crude sulphate (fraction A) was treated exactly according to the conditions of U.S.P. for oxidative removal of brucine and the determination of the associated alkaloid volumetrically. The residue (a.r.) did not contain any basic material, indicating the absence of other bases apart from brucine.

The picrate was prepared by adding picric acid to a dilute aqueous acid solution of fraction A. On standing, the yellow flocculent precipitate rapidly became orange and micro-crystalline. This behaviour is characteristic of brucine. The picrate, after recrystallization from dilute picric acid, did not melt below 270° C. either alone or when mixed with brucine picrate prepared by the same method.

Fraction B was found to consist predominantly of brucine sulphate, with a small amount of non-brucine alkaloid, probably strychnine. This was shown by the following experiments:

The base (B) when prepared in a similar manner to base (A) melted at 167–169° C. (cf. brucine, 178° C.).

0·4 gm. of fraction (B) was treated exactly according to the U.S.P. assay method for oxidative removal of brucine and the associated alkaloid determined volumetrically. The non-brucine residue, weighing 0·04 gm. did not contain any basic material. However, it gave good positive tests for alkaloid, and experiments in mice showed that it was approximately one-third as toxic as strychnine. This indicated that approximately 3 p.c. of fraction B consisted of strychnine.

The aqueous mother liquor (C), after removal of fractions (A) and (B), was adjusted to a pH of about 5 with ammonia, and repeatedly shaken with chloroform until the aqueous extract (D), although containing alkaloid when tested with Mayer's reagent, did not yield the characteristic brucine red coloration with nitric acid. As the first chloroform shaking contained the bulk of the alkaloid, this was treated separately from the others. The first chloroform shaking (E), and the subsequent chloroform shakings (F), were evaporated to dryness, yielding brown resins (fractions E and F respectively). Excess ammonia solution was added to the aqueous extract (D), and the remaining alkaloid extracted with chloroform. The chloroform extract, on evaporation to dryness on the water bath, yielded a viscous resin which set to a light brown brittle varnish on cooling to room temperature (fraction G).

Treatment of Fraction E.

Strychnine was isolated from this fraction by a modification of the method used by Allen and Allport (1940) for the micro chemical separation of strychnine and brucine.

2·0 gm. of the residue were dissolved in 40 ml. of N/5 sulphuric acid, 40 ml. of 1 N oxalic acid and an equal volume of 3 p.c. potassium ferrocyanide solution added, and the whole

allowed to stand for one hour. After this time the solution was filtered, and the ferrocyanide precipitate thoroughly washed with 1 p.c. aqueous sulphuric acid. The precipitate was suspended in ammoniacal aqueous solution (10 p.c.) and the liberated base extracted with chloroform. The chloroform solution was evaporated to dryness and the residue dried at 100° C., yielding crude crystalline strychnine base. This was dissolved in 30 ml. of N/3 aqueous sulphuric acid, and oxidized with nitric and nitrous acid according to the conditions of the U.S.P. for the removal of brucine.

The crystalline alkaloid residue (a.r.) remaining after the removal of brucine was washed with approximately 10 ml. of ethanol, and air dried. (The yield, 1·4 gm., corresponded to a strychnine content of approximately 0·16 p.c.). Confirmation of the identity of the residue (a.r.) as strychnine was indicated by the melting point (260–270° C.), the toxicity in mice (which was of the same order as strychnine), and the characteristic colour reaction with concentrated sulphuric acid and potassium dichromate.

Treatment of Fraction F.

This fraction contained brucine, as indicated by the characteristic brucine red colouration obtained with nitric acid. However, no markedly distinct fractions could be obtained from this fraction when examined by the method outlined above for the isolation of strychnine, brucine, and non-strychnine-brucine alkaloids. All attempts to resolve this into different fractions by fractional solubility and precipitation with solvents and mixtures of solvents proved abortive.

This alkaloid fraction appears to be a mixture of brucine and other alkaloids.

Treatment of Fraction G.

This was purified by reprecipitating several times from acetone with ether. The purified material did not contain brucine, as indicated by the absence of the characteristic brucine red colouration with nitric acid, or strychnine as indicated by the absence of any precipitate with potassium ferrocyanide under the conditions previously outlined for the isolation of strychnine. The toxicity in mice (L.D.50 greater than 250 mg./kg.) further excluded the presence of strychnine (L.D.50 2 mg./kg.) or brucine (L.D.50 about 40 mg./kg.).

This alkaloid fraction has been tentatively called lucidine-S.

Lucidine-S can be isolated as a white amorphous powder by rendering aqueous solutions of its salts alkaline with sodium hydroxide, washing the precipitate with water, and drying in air.

It is very soluble in chloroform and ethanol, very sparingly soluble in ether, benzene, and water and petroleum ether.

It produces heavy precipitates in dilute acid solution with the usual alkaloid reagents.

Concentrated sulphuric acid produces no marked colour change. Concentrated nitric acid produces a pale yellow colouration (cf. brucine, deep red). Concentrated sulphuric acid and potassium dichromate produce a deep red-brown colour (cf. strychnine).

Attempts to prepare a crystalline hydrochloride, sulphate, iodide, and picrate, from aqueous and alcoholic solutions were unsuccessful.

The Isolation of Lucidine-L from the Leaf.

3·5 kg. of finely-ground leaf were treated with 600 gm. of lime, moistened with water, thoroughly stirred, and after drying in air, completely extracted with ether-chloroform (2:1). The ether-chloroform extract was concentrated to a small volume and the alkaloid extracted by shaking with 500 ml. lots of 1 N aqueous hydrochloric acid. The aqueous acid solution was then made alkaline with ammonium carbonate, and shaken gently with chloroform until the

aqueous solution, although containing alkaloid as tested with Mayer's reagent, did not contain brucine as tested with dilute nitric acid. The aqueous solution was then rendered more strongly alkaline with ammonia, and shaken with chloroform. The chloroform extracts were concentrated to dryness, depositing lucidine-L as a clear varnish (H).

Purification of Lucidine-L.

The varnish (H) was digested with hot benzene from which it separated in an amorphous form on cooling. As this gave a faint red reaction with dilute nitric acid, a small portion (0·1 gm.) was oxidized under U.S.P. conditions to ascertain its purity. 0·092 gm. was recovered after oxidation, indicating approximately 8 p.c. brucine present. The separated material was reprecipitated several times from dilute aqueous acid solution with ammonia.

TABLE 2.

Toxicity Table.

Fraction.	(mg./kg.).	Toxicity expressed as No. killed. / No. used.	L.D.50.
Seed sample 1	6·6	0/4	Approx. 10 mg./kg.
	8·25	1/4	
	10·0	2/4	
Seed sample 2	6·6	1/3	
	10·0	3/3	
Seed sample 5	7·5	0/4	Approx. 10 mg./kg.
	10·0	4/7	
Leaf sample 9	6·6	1/6	Approx. 10 mg./kg.
	10·0	3/5	
	13·2	4/5	
Bark sample 10	10·0	1/4	
	13·2	2/2	
Pure strychnine	2·0	6/12	2·0 mg./kg.
Pure brucine	40·0	2/4	Approx. 40 mg./kg.
	50·0	2/2	
Total alkaloid from bulk extraction of seed	15·0	0/3	Between 15 and 25 mg./kg.
	25·0	4/5	
Strychnine from fraction E*	2·0	1/2	2·25 mg./kg.
	2·5	2/2	
Lucidine-S	150·0	0/1	>330 mg./kg.
	330·0	0/1	
Lucidine-L	330·0	0/3	Between 330 and 500 mg./kg.
	500·0	3/3	
Lucidine-L (intraperitoneal)	66·0	0/1	
	160·0	0/1	

* Experiments in rabbits indicated that the L.D.50 of oral doses is approximately the same as strychnine.

The final precipitate was washed thoroughly with water, and air dried, yielding lucidine-L as a creamy amorphous powder.

This was treated similarly to lucidine-S in an attempt to isolate crystalline material. However, all attempts were unsuccessful. It appears to be identical with lucidine-S.

PHARMACOLOGY.

Lucidine-S.

The pharmacology of lucidine-S was restricted to toxicity tests on mice and rabbits (Table 2).

Owing to limited supplies of material, exact determinations were not made, but the results indicate the L.D.50 of intravenous doses in rabbits and mice is greater than 60 mg./kg. and 250 mg./kg. respectively. It may be less toxic as toxic doses in mice produced strychnine-like convulsions, indicating the possible presence of small traces of strychnine impurity.

Experiments with rabbits indicated no marked antagonism between strychnine and lucidine.

Lucidine-L.

Toxicity experiments (Table 2) indicate that the toxicity is of the same order as lucidine-S. Toxic doses produced strychnine-like convulsions. It does not show any marked physiological activity. In anaesthetized cats and dogs (10 mg./kg.) it produces a fall in blood pressure similar to that produced by brucine. It has no action on the isolated heart of a frog (1 : 1,000), nor has it a curare-like action (1 : 1,000). It has no local anaesthetic or mydriatic action, as tested with a 2 p.c. solution on a rabbit's eye.

SUMMARY.

The seed, leaf and bark of *S. lucida* have been examined for the presence of alkaloids. The seeds contain a low percentage of strychnine (approximately 0·3 p.c. from toxicity data), and a high percentage of brucine (1·5–2·4 p.c.), in addition to a non-strychnine-brucine residual alkaloid fraction which has been tentatively called lucidine-S. The percentage of lucidine-S was indicated from biological assay data of the seed. There is in the leaf of *S. lucida*, in addition to strychnine and brucine, another alkaloid fraction (lucidine-L) similar to lucidine-S. The pharmacology of lucidine-S and L is given.

REFERENCES.

Jensen, H. R. (1916): Pharm. J., p. 458.
British Pharmacopoeia, 1932.
United States Pharmacopoeia XI, 1936.
Allen, J. and Allport, N. L. (1940): Quart. J. Pharm. and Pharmacol., 13, p. 252.
Watson, E. M. (1940–41): J. Roy. Soc. W.A., 27, p. 117 (pub. 1943).
Report of Poison Plants Committee of New South Wales, 1942.
Trautner, E. M. and Shaw, F. H. (1945): J. Austral. chem. Inst., 12, p. 232.

THE INFLUENCE OF STORAGE ON THE COAGULATION FACTORS OF HUMAN PLASMA

by P. FANTL AND MARGARET H. NANCE[1]

(From the Baker Medical Research Institute, Alfred Hospital, Melbourne).

(Accepted for publication, 20th December, 1947.)

Previous workers have noted that blood plasma during storage loses its coagulability. Crosbie *et al.* (1941) found that plasma coagulation time measured by simple recalcification decreased in the first twenty-four hours of storage. Apparently this was due to the liberation of thromboplastic substances. In the ensuing period, however, a gradual increase of coagulation time occurred. Prothrombin estimations in aged plasma have given contradictory results, thus Rhoads and Panzer (1939) found a rapid decline of apparent prothrombin activity by the use of the one-stage technique, an observation which was confirmed by all subsequent workers. On the other hand, investigators using the two-stage technique of Warner, Brinkhous and Smith (1936) agree with Lord and Pastore (1939), that a much smaller loss in actual prothrombin concentration takes place during storage. As an explanation for the impaired coagulation of stored plasma, Lavergne *et al.* (1942) suggested that the reactivity of fibrinogen had decreased, an assumption which was supported by Loomis and Seegers (1947). According to Quick (1943) the delay in coagulation is due to the disappearance of an essential factor which he called prothrombin A. Finally, Banfi *et al.* (1945) observed a marked anti-thrombic activity of stored plasma and believed that this phenomenon was responsible for the decline in coagulability. In the course of our own investigations on the stability of prothrombin accelerator a number of human plasma specimens were investigated. From the results obtained it was possible to obtain a clear picture of the factors responsible for the impaired coagulability of stored plasma.

EXPERIMENTAL.

Blood samples were obtained from normal donors. Following addition of 0·1 volumes of 0·1 M sodium oxalate to whole blood and separation of the plasma, the specimens were stored at + 6° C. in test tubes plugged with cotton-wool. Only sterile plasma samples were used. The thromboplastin in the estimation of prothrombin activity by the one-stage technique (Quick, 1938) was a 6 p.c. extract of acetone-dehydrated human brain in 0·15 M saline and was mixed prior to use with an equal volume of 0·01 M calcium chloride. The estimation of prothrombin by a two-stage technique was carried out as described previously (Fantl and Nance, 1948). Thrombin clotting time was measured after the addition of 0·1 ml. of human

[1] Working under full-time grant from the National Health and Medical Research Council.

thrombin to 0·2 ml. of oxalated plasma at 37° C. Prothrombin was separated from oxalated plasma by adsorption on barium sulphate and elution with M/15 Sörensen phosphate buffer pH 7·8. Prothrombin accelerator was prepared from prothrombin-free plasma by ammonium sulphate precipitation. The details for these procedures are given by Fantl and Nance (1948). Fibrinogen and other protein estimations were carried out by a biuret technique as indicated previously (Fantl and Nance, 1947). In Table 1 the results obtained from specimens which had been stored from two to seven weeks are recorded.

TABLE 1.

Comparison of plasma coagulation times before and after storage.

Fresh plasma.			Stored plasma.				
				Prothrombin time in secs.			
Prothrombin time in secs.	Mg. p.c. Fibrinogen.	Thrombin time in secs.	Time of storage in weeks.	Undiluted.	Diluted with human alumina plasma 1:1.	Mg. p.c. fibrinogen.	Thrombin time in secs.
	242	12	2	45	15	228	40
12	274		2½	85	16	293	36
11·5	280		2½	79	16	306	45
12·5	230		2½	90	17·5	246	56
12·5	225		2½	88	17·5	258	67
	305	10·8	4	112	20	298	52
		9·1	6	200	21		95
		10	6	500	22		135
16·5	228		7	170	15·5	238	150
16·5	207		7	110	14·5	206	110
17·5	147		7	127	15	160	91

A marked delay in the coagulation time estimated by the one-stage technique can be observed in all stored specimens, indicating less than 5 p.c. prothrombin activity. Further the thrombin clotting time was markedly prolonged. On the other hand fibrinogen concentration was not altered, and the estimations were carried out by the use of thrombin. The slightly higher values in some of the stored specimens must be attributed to evaporation. In addition the pH of the stored specimens was between 8·6 and 8·8, but the increase in alkalinity has little influence on the coagulation mechanism. The addition of equal volumes of stored plasma to prothrombin-free plasma prepared by treating fresh human oxalated plasma with C-gamma alumina gel or barium sulphate resulted in all cases in the reappearance of prothrombin concentrations between 60 and 100 p.c.

That the anti-thrombin activity of stored plasma can partly explain the delayed coagulation time will be seen from experiments in which the stored plasma was mixed with fresh oxalated plasma.

The results indicate that fresh plasma contains factors which counteract the anti-thrombic activity of stored plasma.

After a storage period of two to three weeks the thrombin inhibitors can be overcome by the addition of fresh plasma. However, anti-thrombic activity in plasma stored for four to

seven weeks is insufficient to explain the prolonged prothrombin time because in the latter instance addition of fresh human plasma did not restore the thrombin clotting time to pre-storage level. Essentially similar results to those tabulated were obtained when instead of fresh plasma, prothrombin-free plasma was used as diluent.

TABLE 2.

*Thrombin clotting times (seconds) of stored human plasma.**

Storage time (days).	Undiluted plasma.	Diluted 1:1 with fresh plasma.	Storage time (days).	Undiluted plasma.	Diluted 1:1 with fresh plasma.
17	36	20	46	47	35
17	45	20	46	45	40
17	56	25·5	46	135	45
17	67	21	49	91	38
26	52	26	49	110	44

* The thrombin clotting time of the plasma used as diluent was 16 seconds.

The assumption that an altered reactivity of fibrinogen in the stored plasma is responsible for the delayed coagulation time was tested in several independent ways. Table 3 shows prothrombin estimations which were carried out with stored plasma specimens diluted with either 0·15 M sodium chloride, prothrombin-free plasma, or freshly prepared solution of human fibrinogen. The latter was prepared from fresh human oxalated plasma from which prothrombin was removed by barium sulphate adsorption. Three successive precipitations with phosphate, according to Jacques (1943), or with 25 p.c. ammonium sulphate, were carried out. The preparations were dialyzed against 0·15 M sodium chloride containing 0·01 M sodium oxalate, at 6° C. for 20 hours. The pH was adjusted to 7·2 with sodium bicarbonate. As judged by clotting with thrombin, the preparations were 97 p.c. pure.

TABLE 3.

Prothrombin times of stored plasma.

Undiluted plasma.	Diluted 1:1 with 0·15 M sodium chloride.	Diluted 1:1 with human alumina plasma.	Diluted 1:1 with human fibrinogen.*	Stored period (days).	Storage temperature.
secs.	secs.	secs.	secs.		
85	59	16	61	17	+6° C.
79	70	16	63	17	+6° C.
90	70	17·5	67	17	+6° C.
88	76	17·5	74	17	+6° C.
240	150			42	+20° C.
∞	1,440	41	960	32	+20° C.
420	315	34	310	21	+20° C.

* The concentration of added fibrinogen was varied from 30 to 60 mg. p.c. fibrinogen nitrogen without affecting the results.

From these results two facts are apparent. Firstly, in contrast to fresh plasma, dilution of stored plasma with isotonic saline produces a shortened prothrombin time. Secondly, dilution with fibrinogen solution gives similar results to those obtained with sodium chloride.

In addition to these experiments fibrinogen was also prepared from stored plasma and tested with several substrates (stored plasma, isolated prothrombin from fresh as well as stored plasma).

TABLE 4.

Influence of fibrinogen prepared from fresh human plasma I and from stored human plasma II on prothrombin activity.

	Tested with:		
	Fibrinogen I.	Fibrinogen II.	Alumina plasma.
	secs.	secs.	secs.
Prothrombin G	37	48	16
Prothrombin H	240	360	34
Stored plasma	225	310	34

Prothrombin G was isolated from fresh human plasma, prothrombin H from human plasma stored for six weeks at 20° C. Fibrinogen I was prepared from fresh plasma as described earlier. Fibrinogen II was prepared from human plasma stored for 12 days at +6° C. The prothrombin activity was measured in the cases of prothrombin G and H by the two-stage technique and the start values are given in the above table. The stored plasma was assayed by the one-stage technique. The fibrinogen concentration and the isotonicity were identical in both instances. The clotting times were longer in the case of fibrinogen isolated from stored plasma. However, if these results are compared with the marked activation by alumina plasma it is apparent that the reaction rate of fibrinogen from fresh or stored plasma has little influence on the prothrombin activity.

TABLE 5.

Influence of prothrombin accelerator on prothrombin time of stored plasma: (one-stage technique).

Undiluted plasma.	Diluted with fibrinogen in saline.	Diluted 1:1 with alumina plasma.	With fibrinogen + accelerator.
secs.	secs.	secs.	secs.
∞	960	41	44
420	310	34	31

Finally, direct evidence that the conversion rate of fibrinogen in the stored plasma has no influence on prothrombin activity was obtained by the addition of isolated prothrombin accelerator to stored specimens.

The prothrombin accelerator was prepared from fresh human plasma. It contained 300 mg. p.c. protein nitrogen and was free of prothrombin and thrombin, but 10 mg. p.c. fibrinogen

nitrogen were present. Since it was shown in Table 3 that much larger amounts of added fibrinogen are not able to restore the prothrombin activity it is certain that the reappearance of prothrombin activity is due to the presence of prothrombin accelerator.

Finally, the globulin fraction containing prothrombin accelerator was prepared from human plasma stored for varying periods and was tested on several prothrombin preparations.

TABLE 6.

Influence of storage on stability of prothrombin accelerator. (Estimated by the two-stage technique):

	With fibrinogen in saline.	With fibrinogen and accelerator.	Storage time and temperature of plasma from which accelerator was prepared.	With fresh alumina plasma.
Prothrombin E Prothrombin E	145″ —″	15″ 39″	Nil 17 days, 6° C.	18″
Prothrombin F	240″	196″	28 days, 6° C.	25″
Prothrombin D	220″	230″	28 days, 20° C.	39″
Prothrombin C	90″	96″	28 days, 20° C.	25″

The prothrombin preparations (C-F) were isolated from plasma specimens stored for periods of 17 days at +6° C. to 35 days at +20° C.

The results indicate that it is possible to isolate prothrombin accelerator from plasma stored for two to three weeks at +6° C. After four to five weeks storage at +20° C., however, the globulin fraction loses its accelerating properties.

DISCUSSION.

The experiments reported in this paper were carried out with the isolated factors of the coagulation mechanism. Since material from human sources only was used, the interpretation requires few assumptions. The techniques employed were such as to guarantee high purity of the components. The procedure of separation of prothrombin from plasma can be considered satisfactory, since the potency of the isolated fractions was followed by measurement of activity and protein nitrogen estimations. No unaccounted loss during barium sulphate adsorption and phosphate elution was found, indicating that the isolation procedure did not involve any structural changes. In spite of the fact that stored plasma has a delayed prothrombin time when measured by Quick's technique, prothrombin was isolated in potency much greater than anticipated. Loomis

and Seegers (1947) using a different technique have reported similar findings with beef plasma. The changes attributed to fibrinogen during storage have occupied the attention of several investigators. Although Taylor *et al.* (1944) found no difference in fibrinogen concentration in plasma stored for two to three months at room temperature, Loomis and Seegers believed that the conversion rate had diminished because beef fibrinogen added to stored plasma restored the coagulability to the pre-storage level. It can be shown, however, that purified fibrinogen is not able to do so. Deutsch and Gerarde (1946) have also been unable to substantiate Loomis and Seegers' claim. The partial restoration of the prothrombin time following addition of fibrinogen solution has been shown to be equal to that observed by dilution with 0·15 M sodium chloride (Table 3). This is apparently due to the counteracting effect of dilution on thrombin inhibitors which develop during storage. Fibrinogen isolated from stored plasma did show a different reaction rate when tested on isolated prothrombin, but the differences between fibrinogen isolated from fresh and aged plasma are not detectable in Quick's procedure. Quick (1943) assumes that an essential component of the prothrombin molecule, called prothrombin A, disappears during storage.[2]

From the study of isolated prothrombin both from fresh and stored plasma it could be shown, however, that prothrombin is the only precursor of thrombin (Seegers, Loomis and Vandenbelt, 1945 and Fantl and Nance, 1946). However, normal plasma contains in the globulin fraction, a factor capable of accelerating thrombin formation, Fantl and Nance (1948), and this principle has been isolated by Owren (1947) and termed Factor V. Ware, Guest and Seegers (1947) confirmed the presence of prothrombin accelerator in beef plasma. The speed of thrombin formation is dependent, in addition to prothrombin, upon the concentration of prothrombin accelerator. In contrast to other mammalian plasma, the activity of prothrombin accelerator in human blood is insufficient for rapid thrombin formation even under normal conditions. A slight fall will therefore produce a marked delay in the prothrombin time when measured by the one-stage procedure which is further accentuated by the development of thrombin inhibitors.

[2] Honorato (1947) designated prothrombin A, plasmatic co-factor of thromboplastin, whilst Honorato and Quick (1947) recently renamed it "labile factor." Munro and Munro (1947) believe now that the prothrombin molecule contains a labile group which is inactivated by storage and can be reactivated by some other protein present in plasma.

SUMMARY.

The factors responsible for the decline in coagulability of stored human plasma are: firstly, an increase of thrombin inhibitors, and secondly, a gradual loss of prothrombin accelerator, a process which is complete after four weeks at 20° C. A much smaller loss of prothrombin occurs during this period. No change in fibrinogen concentration takes place and any altered reactivity of fibrinogen is not detectable in the estimation of prothrombin by a one-stage procedure.

Acknowledgments. We wish to thank Miss J. Tolhurst, M.Sc. for carrying out sterility tests on the stored plasma, and Mrs. S. E. Simon, B.Sc. for the preparation of fibrinogen solutions.

REFERENCES.

Banfi, R. F., Tanturi, C. A. and Bay, R. (1945): J. lab. clin. Med., 30, p. 512.
Crosbie, A., Scarborough, H. and Thompson, J. C. (1941): Edin. med. J., 48, p. 41.
Deutsch, H. F. and Gerarde, H. W. (1946): J. biol. Chem., 166, p. 381.
Fantl, P. and Nance, Margaret H. (1946): Nature, 158, p. 708.
Fantl, P. and Nance, Margaret H. (1947): Austral. J. exp. Biol., 25, p. 95.
Fantl, P. and Nance, Margaret H. (1948): Med. J. Austral., I, p. 128.
Honorato, R. (1947): Amer. J. Physiol., 150, p. 381.
Honorato, R. and Quick, A. J. (1947): Amer. J. Physiol., 150, p. 405.
Jaques, L. B. (1943): Biochem. J., 37, p. 344.
Lavergne, G. H. and Lavergne-Poindessault, B. L. (1942): Compt. rend. Soc. Biol., 136, p. 445.
Loomis, E. C. and Seegers, W. H. (1947): Amer. J. Physiol., 148, p. 563.
Lord, J. W., Jnr. and Pastore, J. B. (1939): J. Amer. med. Assoc., 113, p. 2,231.
Munro, M. P. and Munro, F. L. (1947): Amer. J. Physiol., 150, p. 409.
Owren, P. A. (1947): "The Coagulation of blood. Investigations on a new Clotting Factor," Oslo.
Quick, A. J. (1943): Amer. J. Physiol., 140, p. 212.
Quick, A. J. (1938): J. Amer. med. Assoc., 110, p. 1,658.
Rhoads, J. E. and Panzer, L. M. (1939): *Ibid.*, 112, p. 309.
Seegers, W. H., Loomis, E. C. and Vandenbelt, J. M. (1945): Arch. Biochem., 6. p. 85.
Taylor, F. H. L., Lozner, E. L., Davidson, C. S., Tagnon, H. J. and Newhouser, L. R. (1944): J. clin. Invest., 23, p. 351.
Ware, A. G., Guest, M. M. and Seegers, W. H. (1947): J. biol. Chem., 169, p. 231.
Warner, E. D., Brinkhous, K. M. and Smith, H. P. (1936): Amer. J. Physiol., 114, p. 667.

THE LIBERATION OF RENIN FROM THE KIDNEY BY TISSUE INJURY WITH COBRA VENOM

by G. REID AND H. A. JENKINS[1]

(From the Physiology Department, University of Melbourne).

(Accepted for publication, 12th December, 1947.)

It is well established that renal ischaemia in both man and animals causes the liberation, from the kidneys, of renin; and that the effect of this substance, in the early stages at least, can account for the hypertension which in experimental animals usually follows the ischaemia.

In human pathology, hypertension is commonly associated with renal lesions; but it is exceptional to find that such lesions simply consist of organic vascular obstruction in the kidney circulation. Very often when organic vascular changes can be found they are not necessarily confined to the kidney and they are believed to be secondary to the hypertension which may or may not be renal in its origin. Likewise, we have no definite evidence that essential hypertension is preceded by functional renal vaso-constriction. On the other hand, when hypertension clearly is secondary to a renal lesion such as nephritis, a significant change in the kidney is damage to the glomeruli and tubules. It is interesting also to note that it is only in phases of acute damage of the kidney that it has been possible to demonstrate renin in the blood of human hypertensives (Dexter and Haynes, 1944). While it must be borne in mind that renin liberation in these conditions may result from ischaemia due to the mechanical effect of the inflammatory process, there is also the other possibility that tissue injury by the unknown causal agent may be responsible.

This paper reports experiments to determine whether renin can be liberated from the kidney by tissue injury other than that which may be produced by ischaemia.

METHODS.

Ideally it would be desirable to use as a damaging agent the one actually responsible for nephritis; but since this is hypothetical it was decided to use cobra venom because in addition to its being a very potent agent, its own vascular effects are already well known (Feldberg and Kellaway, 1937a and 1937b).

Cats were used. Briefly, one or both kidneys were left *in situ* in eviscerated adrenalectomized animals and the venom was injected into the arterial supply of the kidneys. The effect on the animal's own blood pressure was noted, or blood from the renal vein was injected or

[1] Technical assistance was provided by a grant from the National Health and Medical Research Council.

cross-circulated into another animal and the effect on its blood pressure observed, or blood from the renal veins was assayed for renin by the method of Leloir *et al.* (1940).

In more detail, the cats were anaesthetized with chloralose, the vagi divided and, usually, artificial ventilation was supplied. The cat was eviscerated and both suprarenal glands removed. Sometimes the left kidney was removed. The aorta was tied below the renal arteries and a metal cannula inserted into the cut stump of the superior mesenteric artery.

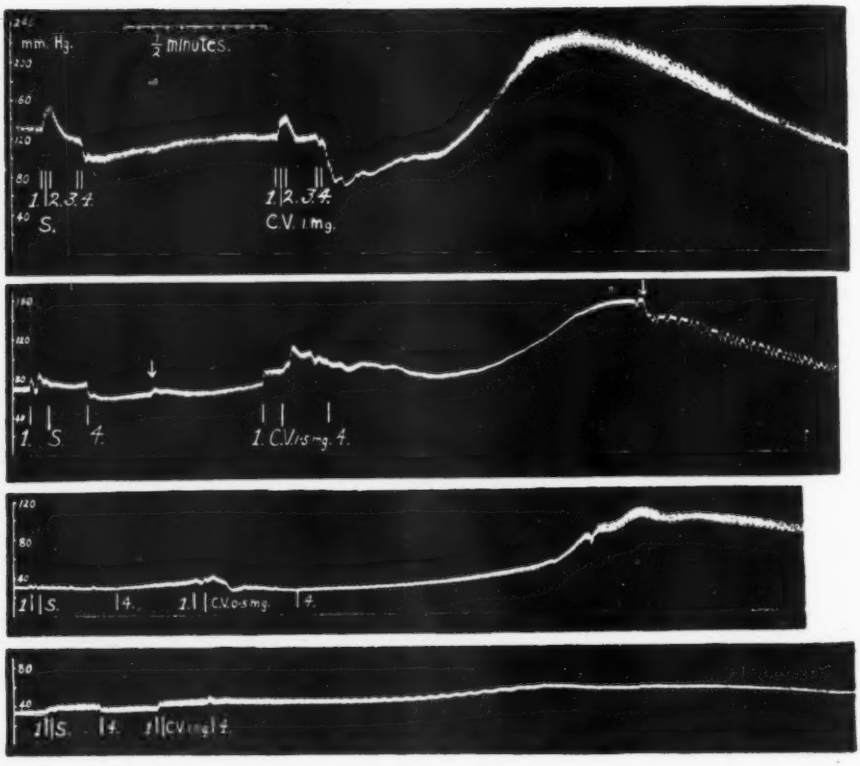


Fig. 1. Blood pressure record of four cats prepared as described in the text. At the arrows in the second figure, 4·0 ml. of blood were removed for injection into another cat.

Before injecting the venom through this cannula the aorta was occluded above the origin of the superior mesenteric artery. By this means the venom was injected mainly into the kidney and kept there until the clip on the aorta was released a few minutes later. Before injecting the venom a control procedure was done consisting of occlusion of the aorta and injection of normal saline. The blood pressure was recorded from a carotid artery. The liberation of a pressor substance was detected by observing the effect on the animal's own blood pressure or on that of another animal cross-circulated with the first (see Results). In other experiments

2–5 ml. of blood were withdrawn from the caval pocket into a syringe containing heparin and immediately injected into another non-eviscerated animal whose blood pressure was recorded. The renin content of renal blood serum was also estimated in a roughly quantitative fashion by the method of Leloir *et al.* (1940), as described below:

About 10 ml. of serum were taken and the hypertensinogen and hypertensinase content destroyed by incubation at 37° C. for 30 minutes after adjusting the pH to 3·7–3·9 (glass electrode) with 2M phosphoric acid. After readjusting the pH to 7·0 a volume corresponding to 10 ml. was incubated with 6·0 ml. of a solution of hypertensinase-free hypertensinogen (prepared from horse serum) for 2–4 hours at 37° C. (1/10,000 merthiolate being added as a preservative).

After incubation, three volumes of warm (40° C.) absolute alcohol were added and the mixture allowed to stand for one hour and filtered. The residue left after this filtrate had been evaporated to dryness under reduced pressure, was reconstituted in 6·0 ml. of saline. This solution was tested for its pressor activity by injection into cats or rabbits under chloralose anaesthesia. (See Fig. 4).

When the left kidney was removed before the experiment, the renin content of both was extracted by the method of Pickering and Prinzmetal (1937).

Control experiments were performed in which cobra venom was injected intra-arterially into the hind limb of eviscerated cats. (See Results).

RESULTS.

In eight cats cobra venom in doses ranging from 0·3 to 2·0 mg. was injected initially into the arterial supply of one or both kidneys. In seven additional cats cobra venom was injected initially into the arterial supply of the hind limb; and in five of these animals it was subsequently injected intra-arterially into the kidneys.

Injections into the Kidney.

In six out of the eight cats into which cobra venom was injected initially into the vascular supply of the kidney a rise of blood pressure took place; this began 4·5–7 minutes after the circulation was released, ranging in magnitude from 15–100 mm. of Hg., and lasted from 10–20 minutes. The results of four of the experiments are illustrated in Fig. 1. At 1 the aorta was clamped above the renal vessels, at 2 and 3 (upper panel only) the inferior vena cava was occluded and released respectively, at 4 the aorta was released and at the longer strokes a control injection of normal saline or the indicated amount of cobra venom was injected. In three experiments including the ones in which no pressor response occurred 2–5 ml. of blood were withdrawn from the inferior vena cava 4–15 minutes after the circulation was released (a) following the control injection and (b) following the injection of venom. In each case the sample (b) when injected into another animal gave a pressor response which was not given by sample (a).

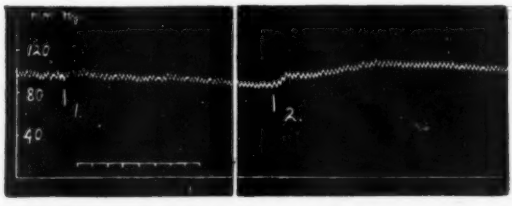


Fig. 2. Blood pressure record from a non-eviscerated cat under chloralose anaesthesia. At 1 and 2 blood from another animal was injected intravenously. See text for details.

This is illustrated by Fig. 2 which shows the effect of the intravenous injection of two such samples (a) and (b) each 3 ml. in volume at 1 and 2 respectively. In this experiment the first cat which was injected with 1·0 mg. of cobra venom had itself showed no pressor response.

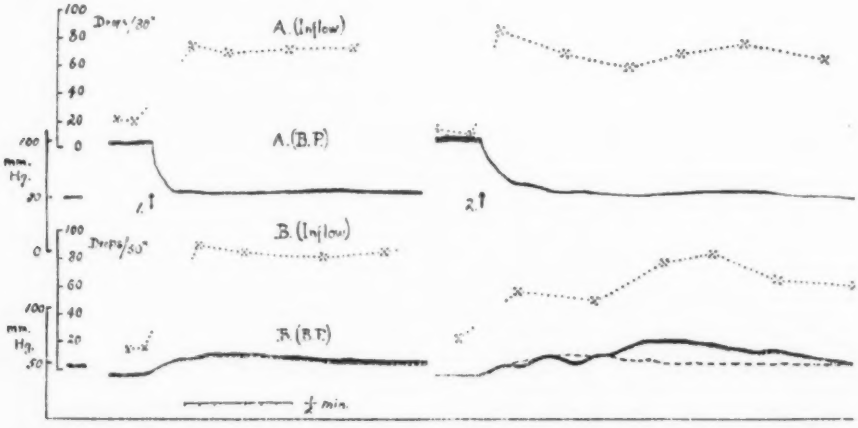


Fig. 3. Cross circulation experiment with two eviscerated cats. The upper part of the Figure shows the blood pressure of "A" and the inflow from "B" to "A"; the lower part of the Figure shows the blood pressure of "B" and the inflow from "A" to "B". See text for details.

That a humoral agent is responsible for the pressor effect was also indicated by the results of a cross-circulation experiment illustrated in Fig. 3. For this experiment two cats were eviscerated and set up as already described and in addition the inferior vena cava of each was connected by rubber tubing to an external jugular vein of the other. The aorta of the donor (A) was occluded for 5 minutes; this resulted in a rise of blood pressure in it, and a greatly reduced inflow from (A) to the recipient (B) (B inflow) whose blood pressure consequently fell. On releasing the aorta at the first arrow (the latter part only, of the occlusion period being shown) the inflow to the recipient increased and within 6 minutes the blood pressure and inflows had returned to their original level. Between the two panels the aorta was once again occluded, 0·75 mg. of cobra venom injected, and after five minutes the aorta was released at the second arrow. Up to this point the blood pressure changes in both donor and recipient were essentially the same as during the control phase; but now after the release of the aorta the pressure in (A) fell somewhat less rapidly and the inflow to the recipient (B inflow) remained at a low level for some minutes indicating vascular obstruction in the kidney. Nevertheless, despite the decreased inflow to the recipient relative to that found in the same phase of the control period the blood pressure of the latter actually rose some 30 mm. of Hg. higher. To facilitate comparison with the control the mean blood pressure during that phase has been shown as a dotted line in the second panel.

Control Injections of Cobra Venom into the Hind Limb.

For these experiments the cat was eviscerated as before and the suprarenal glands removed. The venom was injected into the vascular supply of one hind limb (and pelvis) through the cut stump of an external iliac artery, the aorta being occluded above the origin

of the iliac vessels for some minutes after the injection. After the aorta was released in not one of the seven animals did a rise of blood pressure occur. After this, in five of the animals, the aorta was tied off below the renal arteries which were then released and a further experiment was made by injecting venom into the renal circulation. This procedure caused no rise of pressure in three of the five animals and gave a doubtful response in the other two. This is possibly because of the deterioration of the animal's condition following the hind limb injection. However, in one of these experiments blood samples were taken from the inferior vena cava (a) after the circulation to the injected hind limb was released, (b) after the aorta was tied off below the renal vessels and the renal arteries which had been occluded for 20 minutes were released, and (c) after release of the circulation to the injected kidney. These samples were injected into a second cat; sample (a) had no pressor action, sample (b) a slight one, and sample (c) a marked one.

Identification of Pressor Agent as Renin.

The experiments so far indicate that a pressor agent is liberated from the kidneys but not the hind limb in tissue injury with cobra venom. It was necessary to show that the pressor substance behaved like renin. To show this blood was taken from the caval pocket and the serum, after freeing it from hypertensinase and hypertensinogen, was incubated with equine hypertensinase-free hypertensinogen, and the product tested in the cat for pressor activity.

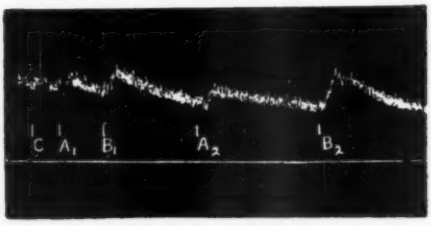


Fig. 4. Rabbit under chloralose anaesthesia. Blood pressure record. Titration of hypertensin formed by the incubation of hypertensinase-free serum with hypertensinogen. See text for details.

In such an experiment a sample of blood taken before the experiment began had no detectable renin; a sample removed 3 minutes after a control occlusion which lasted 10 minutes had slight activity and a third sample taken five minutes after the injection of cobra venom had considerable activity. For the last sample the circulation had been occluded for only 2 minutes. Fig. 4 illustrates such a titration. The product prepared from the sample removed after the control occlusion was injected at A into a rabbit under chloralose anaesthesia, and the corresponding product after venom was given, was injected at B. The figures refer to millilitres of the product injected. Saline was injected at C.

Changes in the Kidney Following the Injection of Venom.

At the conclusion of an experiment the kidney was swollen and congested, and microscopic examination showed hyperaemia and a hyaline exudate in the glomerular capsule and tubules together with extravasated red blood corpuscles. The tubules were dilated and displayed damaged epithelium.

The method which we used for the assay of renin in the kidneys was inadequate to detect a difference between the renin content of an envenomed kidney and its corresponding normal kidney which had been previously removed. The results of all such experiments were indeterminate.

DISCUSSION.

In the past, many methods have been used to produce renal damage. Even before the work of Goldblatt, several attempts had been made to bring about hypertension by means of some sort of interference with the kidney. Much of this earlier work is reviewed by Janeway (1913), and by Goldblatt himself (1934). The methods used included partial or complete occlusion of renal arteries or veins, the production of renal embolism with paraffin or lampblack, partial nephrectomy, and the use of various nephrotoxic agents; the latter has been extensively reviewed by Horn (1937). All of the workers who have been interested in experimental nephropathies have not been concerned with the production of hypertension, but generally it can be said that, until the experiments of Goldblatt, hypertension was an irregular result of the various procedures adopted.

Friedman and Katz (1938) were interested in the problem of the relationship of renal insufficiency to hypertension. They damaged the kidney of the dog by injecting trypsin into the renal vessels. They found that a rise of blood pressure was present in the acute stage only, i.e. in the first one or two days; after that the blood pressure returned to normal levels and remained so for up to three months, although considerable renal tissue had been destroyed and marked renal insufficiency had developed. It would appear as though hypertension occurred during the phase of active damage or ischaemia, but, when the renal tissue had been completely destroyed, renal insufficiency but not hypertension developed. These observers do not deal with the cause of the acute hypertension; they refer to the possibility of its reflex origin or to the liberation of a pressor substance resulting either from the renal damage caused directly by the toxin, or from the concomitant ischaemia.

Our results show that a pressor response occurs in acute experiments when cobra venom is injected into the arterial supply of the kidney. This effect is not due to the action of the venom itself because it does not occur when cobra venom is injected intravenously (Feldberg and Kellaway, 1937a, 1937b). Moreover, a pressor response of this type never occurred when the venom was injected into the hind limb in either the "intact" (Feldberg and Kellaway) or in the eviscerated animal. Our results show, in fact, that a humoral or pressor agent is liberated from the kidneys and its presence can be demonstrated in the renal venous blood even when no pressor response occurs in the injected animal. It can not be demonstrated when venom is injected into the hind limb. Finally, the agent in the renal venous blood was shown to act on hypertensinogen to produce the active pressor substance; in other words, it behaves like renin and can be estimated as such by the method of Leloir *et al.*

Many observers have used periods of complete ischaemia lasting several hours in order to produce, in acute experiments, a rise of blood pressure on releasing the circulation (references to be found in the paper by Quinby *et al.*, 1945). Other observers have used shorter periods; for example, Collins and Hamilton (1940) found pressor responses in the cat after ischaemia, lasting

30 minutes. Quinby, Dexter, Sandmeyer and Haynes (1945) found for both humans and dogs, that complete renal ischaemia of one kidney for 12 minutes caused no pressor response although renin could be detected in the renal venous plasma. These experiments suggest that the very short periods of complete or partial ischaemia which were used in our experiments were unlikely to be solely responsible for the pressor responses which occurred. Moreover, the experiments themselves indicate that the pressor effect of cobra venom is not due to the renal ischaemia, because comparable or longer periods of ischaemia were much less effective. For example, in one experiment the renin content of blood from the renal vein, after 10 minutes' complete occlusion, was very much less than in that removed 5 minutes after the injection of venom, during which time the circulation had only been completely occluded for two minutes.

Has this sort of result any bearing on the hypertension associated with acute nephritis? Renin has been detected in the venous blood of patients in whom the hypertension has been severe and explosive in onset, viz., in acute nephritis, eclampsia and pre-eclampsia, in contradistinction to those with chronic nephritis or essential hypertension (Dexter *et al.*, 1944). Our experiments lend support to the idea that tissue injury as distinct from ischaemia may be a factor in such conditions. The detection of renin in such patients does not necessarily show that a humoral mechanism is the only one concerned with the genesis of hypertension. There is some evidence to indicate that hypertension associated with other kinds of renal damage may have a nervous basis, as for example that due to oxalates or nephrotoxic serum (Arnott and Kellar, 1935, 1936). Pickering (1936) has shown by plethysmographic methods that in human subjects the hypertension of acute nephritis depends on nervous vasoconstriction.

The results which we have described may have been more impressive had it been possible to obtain much more prolonged rises of blood pressure and to detect renin throughout such periods of hypertension. The large doses of venom which it was necessary to use for such acute experiments possibly militated against this; so that we were dealing not only with the pressor effect of renin but also with the depressor effect of cobra venom. A further point is, that commonly in tissue injury not only are active substances liberated but also the enzymes which effect their destruction. Apart from this there may be many substances liberated in addition to renin which may be depressor in action, so that perhaps it is surprising that a pressor effect was obtained at all.

We do not know whether the agent responsible for nephritis more or less selectively liberates renin relative to these other substances, but in view of the derangement of the excretory functions of the renal cells this appears to be unlikely. On the other hand it seems that partial ischaemia acts at such a "level" of cellular make-up that renin is liberated without there being associated damage to the biochemical mechanisms concerned with the better recognized functions of the kidney. Whether tissue injury or ischaemia is the more important in nephritis or eclampsia one cannot say, but in acute injury with cobra venom the tissue injury appears to be the more important.

SUMMARY.

In the eviscerated adrenalectomized cat, cobra venom injected into the renal circulation causes a liberation from the kidney of renin. This is attributable mainly to the direct action of the venom and not to the associated partial ischaemia. The renin may cause a pressor response in the injected animal or may be detectable in the renal venous blood even when no pressor response occurred in the animal actually injected with venom.

REFERENCES.

Arnott, W. M. and Kellar, R. J. (1936): J. Path. and Bact., 42, p. 141.
Arnott, W. M., Kellar, R. J. and Mathew, G. D. (1937): Edin. med. J., 44, p. 205.
Collins, D. A. and Hamilton, A. S. (1940): Amer. J. Physiol., 130, p. 784.
Dexter, L. and Haynes, F. W. (1944): Proc. Soc. exp. Biol. and Med., 55, p. 288.
Feldberg, W. and Kellaway, C. H. (1937a): Austral. J. exp. Biol., 15, p. 159.
Feldberg, W. and Kellaway, C. H. (1937b): *Ibid.*, 15, p. 441.
Friedman, M. and Katz, L. N. (1938): J. exp. Med., 68, p. 485.
Goldblatt, H., Lynch, J., Hanzal, R. F. and Summerville, W. W. (1934): *Ibid.*, 59, p. 347.
Horn, H. (1937): Arch. Path., 23, pp. 71 and 241.
Janeway, T. C. (1913): Amer. J. med. Sci., 145, p. 625.
Leloir, L. F., Munoz, J. M., Braun-Menendez, E. and Fasciolo, J. C. (1940): Rev. de la Soc. Argent. de Biol., 16, p. 635.
Pickering, G. W. (1936): Clin. Sci., 2, p. 363.
Pickering, G. W. and Prinzmetal, M. (1937–38): Clin. Sci., 3, p. 211.
Quinby, W. C., Dexter, L., Sandmeyer, J. A. and Haynes, F. W. (1945): J. clin. Invest., 24, p. 69.

STUDIES ON A FLUORESCENT SUBSTANCE PRESENT IN PLANTS.

PART 3: THE DISTRIBUTION OF SCOPOLETIN IN TOBACCO PLANTS AND SOME HYPOTHESES ON ITS PART IN METABOLISM [1,2]

by RUPERT J. BEST

(From the Waite Agricultural Research Institute, University of Adelaide).

(Accepted for publication, 24th December, 1947.)

A substance which fluoresced in ultra-violet light was found in tobacco and other plants which were infected with tomato spotted wilt virus, and in *Nicotiana glutinosa* plants infected with tobacco mosaic (Best, 1936). The substance was subsequently isolated and identified as 6-methoxy-7-hydroxy 1:2 benzo-pyrone (scopoletin) (Best, 1944).

Although the substance had not previously been recorded as a constituent of tobacco plants, the author has isolated it from normal, healthy tobacco plants. The nature of the fluorescence is very characteristic and the fluorescent light changes colour with change in pH value. Using this character of the fluorescence and its colour under acid and alkaline conditions as a test for its presence, the author has made a qualitative survey of a number of plants and has found that the substance is much more widely distributed than would be gathered from the literature (see e.g. Klein, 1932).

Nothing seems to be known about the part taken by this substance in the normal metabolism of the healthy plant. Chemically, scopoletin is closely related to phenyl acrylic derivatives such as coniferyl alcohol and aldehyde, which in turn are associated with lignin, and to the tannin of tobacco leaf (chlorogenic acid). There are a number of metabolic processes in which a lactone of phenyl propane derivatives could take a direct part or in which it might be produced as a by-product.

The present paper contains a description of the distribution of the substance in the tobacco plant and some hypotheses in relation to its formation and possible part in metabolism.

DISTRIBUTION OF SCOPOLETIN IN TOBACCO PLANTS.

Leaves of tobacco plants which have been lightly wiped with a solution of T.S.W. virus develop localized necrotic lesions three or four days later. When viewed under filtered ultra-violet light (3,000 Å to 4,200 Å) the necrotic portion of the lesion is seen to be surrounded by

1 Paper presented at the Perth 1947 meeting of the Aust. and N.Z. Assoc. Adv. Sci.

2 Glasshouse facilities and part of the cost of these investigations were provided by the Council for Scientific and Industrial Research.

a blue fluorescent halo. This halo is due to an accumulation of scopoletin. It is concentrated in what appears to be the normal mesophyll leaf tissue at the site where the virus is actively multiplying. At a later stage, especially when the lesions have spread to the veins these also become highly charged with scopoletin and finally the roots become strongly fluorescent.

When such a leaf is pressed between sheets of blotting paper a fluorescent outline of the vascular system and the lesions appears on the paper. When a control (healthy) leaf is treated similarly there are usually no fluorescent markings on the paper except at the base of the petiole. Examination of hand sections under a microscope confirms the presence of the substance in the vascular system of both healthy and infected plants.

That more of the substance is present in the virus-damaged plants than in healthy ones is shown not only by the blotting paper traces, but also by the fluorescence of water extracts of the leaves or other parts. Extracts from the virus-injured plants must be diluted many-fold to bring the intensity of the fluorescence down to that of extracts of healthy plants.

The conclusion that the substance is transported in the vascular system is based on the evidence of the blotting paper traces, on macroscopic and microscopic examination of hand sections and on the observation that the enhanced fluorescence of the vascular system occurs only after the haloes in the mesophyll tissue (around the primary lesions) have become established.

TABLE 1.

Showing the effect of age of healthy tobacco plants on the distribution of scopoletin within them as indicated by the intensity of the characteristic fluorescence in ultra-violet light.

	Age of plant (weeks after germination).	Stage of growth No. of leaves besides cotyledens and buds.	Intensity of fluorescence (caused by scopoletin).
A	2	3	Roots, hairs of stem and leaves bright; midribs and base of bud—faint.
B	5	3	Roots and hypocotyl bright. Rest as for A.
C	6	4	As for B but a third of the roots are brightly fluorescent and the rest moderately so.
D	7	6	As for C, but a *smaller* proportion of roots fluoresce brightly.
E	12	9	As for D but very few roots fluoresce brightly. Details as in first column of Table 2.
F	(2½ ft. tall)	20	About 70 p.c. of roots brightly fluorescent. Fluorescence bright all the way up to and including bud. Fainter (but present) in every leaf in bud. Roots, midribs and vascular system chief location.

The maximum amount of fluorescent material is formed when the type of injury is intermediate between a severe localized necrosis and the comparatively slight injury caused by a systemic infection without visible necrosis. For example no fluorescence (of the type given by scopoletin) has been observed in nasturtium leaves which are systemically infected by a mild strain of tomato spotted wilt. This strain produces a non-necrotic mosaic on nasturtium whereas on tobacco it causes visible lesions around which bright fluorescent haloes develop. The virulent strain of T.S.W., however, causes necrotic lesions on nasturtium leaves also and these lesions are surrounded by the characteristic blue fluorescent haloes of scopoletin. That the amount of scopoletin produced by virus injury is so much greater in tobacco than in nasturtium is presumably due to differences in the host plants.

TABLE 2.

Showing the distribution of scopoletin in tobacco plants, as estimated by the intensity of its characterstic fluorescence in ultra-violet light.

Position on plant.	Healthy plants (untrimmed).	T.S.W. (trimmed).
T.S. Stem (near first pair leaves)		
Epidermis and hairs	faint	+
Cortex	faint	+ (gradient)
Pith	faint	+ (gradient)
Endodermis (internal and external)	bright	+ *brilliant*
Phloem (internal and external)	absent	faint to absent (unless plant is saturated)*
Xylem	absent	absent†
Cambium	absent	absent
T.S. Stem (near growing tip)		
Epidermis and hairs	faint	
Cortex	absent (red fluorescence of chlorophyll)	
Pith	absent	
Endodermis (internal and external)	bright	
T.S. Root Stock		
Cortex	bright	
External endodermis	bright	
Xylem	absent	
T.S. Small Roots	fluorescent throughout	
T.S. Leaf (midrib)		
Endodermis	bright	
Phloem	patchy	
Xylem	absent	

Notes. 1. Under high power the fluorescence of individual cells is seen to be peripheral except in the endodermis.

2. In general the fluorescent ring associated with the internal endodermis was more diffuse than that of the external endodermis.

* A few weeks after infection the whole stem became "saturated" with scopoletin in which case all cells fluoresced. The cambium was the last part to be affected.

† i.e. absent unless the stem is "saturated" in which case the medullary rays fluoresce and the protoxylem contains more scopoletin than the later xylem. The xylem, incidentally, has its own yellow fluorescence.

Another example is the production of scopoletin around the necrotic lesions of tobacco mosaic on *Nicotiana glutinosa* whereas fluorescent patches can be found only with great difficulty in tobacco plants systemically infected with the same virus and even then they are faint.

Mechanical injury to tobacco plants has resulted in the development of localized patches of enhanced fluorescence but the order of this increase is nothing like that associated with injury by virus and may have the same or a different explanation.

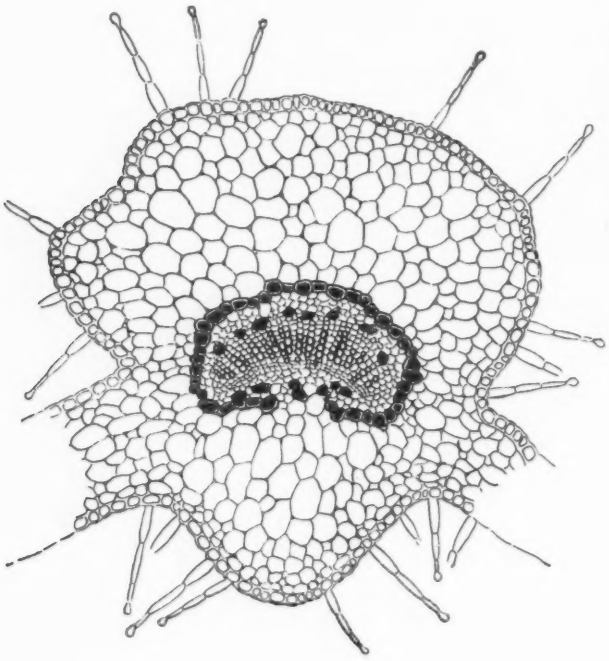


Fig. 1.

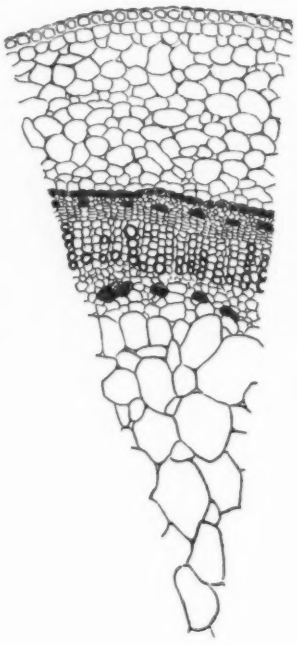


Fig. 2.

Fig. 1. Transverse section of the midrib of a healthy *Nicotiana tabacum* plant. Cells in which scopoletin is most concentrated are blocked in. A second distinct but more diffuse ring of bright fluorescence was located about the internal endodermis.

Fig. 2. Transverse section of the stem of a healthy *Nicotiana tabacum* plant.

(Remarks in legend to Fig. 1 also apply here.)

The distribution of scopoletin as estimated by its fluorescence in healthy tobacco plants at various stages of growth are set out in Table 1 and the distribution of the substance in different parts of the plant in health and disease are set out in Table 2.

Briefly, scopoletin occurs in normal healthy tobacco plants from the time they germinate until they reach maturity. The amount present seems to fall off during the rapidly growing stage and to rise to its greatest concentration as the plant ages. Normally, the

substance is present in the epidermis, hairs, cortex and pith but only as a trace. The xylem and cambium do not normally reveal its presence and the phloem only in trace amounts if at all. Both external and internal endodermis fluoresce brightly under all circumstances.

Infection of a growing plant with the virus of T.S.W. increases the concentration of scopoletin throughout the plant and although the relative distribution is as just described for the healthy plant, the infected plant eventually becomes saturated so that all tissues fluoresce. There would appear to be a definite concentration gradient outward through the cortex from the external endodermis and inward to the pith from the more diffuse internal endodermis. Figs. 1 and 2 are camera lucida drawings of transverse sections of a midrib and of a stem of a young, healthy tobacco plant. (I am indebted to my wife for these drawings.)

When healthy plants have their tops removed, the scopoletin concentration rises, especially in the roots, and a state of affairs between the virus infected and the untopped healthy plant is set up.

DISCUSSION.

A consideration of the possible chemical transformations which could result in the production of scopoletin has suggested the following hypotheses.

The Broken Chain (Diversion of Metabolism) Hypothesis.

Recent work on lignin indicates that coniferyl alcohol and its derivatives are important elements in the structure of that substance. It is therefore quite possible that coniferyl alcohol (or a derivative) normally undergoes a further sequence of changes ending in the lignin molecule and that the virus activity breaks this chain just above coniferyl alcohol. Oxidizing conditions and release of oxidases from injured tissues would lead to the formation of the corresponding acid (ferulic acid) and further enzymic action could result in the formation of the orthohydroxy acid which by subsequent ring closure could form the lactone, scopoletin. All these reactions commonly occur in normal plant metabolism and the final formation of the lactone has a close analogue in the formation of umbelliferone from umbellic acid.

The author has in fact demonstrated the presence of tyrosinase in tobacco leaves infected with T.S.W. virus (Best, 1937).

The proposed sequence of changes (on this basis) is set out schematically in Fig. 3 (I to IV).

If Hibbert's (1942) statement "the universal occurrence of coniferyl alcohol in all young plant tissue" is correct, the conditions of the sequence I to IV are present in the tobacco leaf.

The Decomposition of Lignin Hypothesis.

In a letter to the author, Andreae (1947) stated that he had isolated scopoletin from virus-infected potato plants (first year infection), using the author's method. Andreae suggested that the scopoletin may be formed as a result of the breakdown of lignin to coniferyl alcohol and thence by way of II and III to IV. While this is quite possible, the evidence seems to be against it

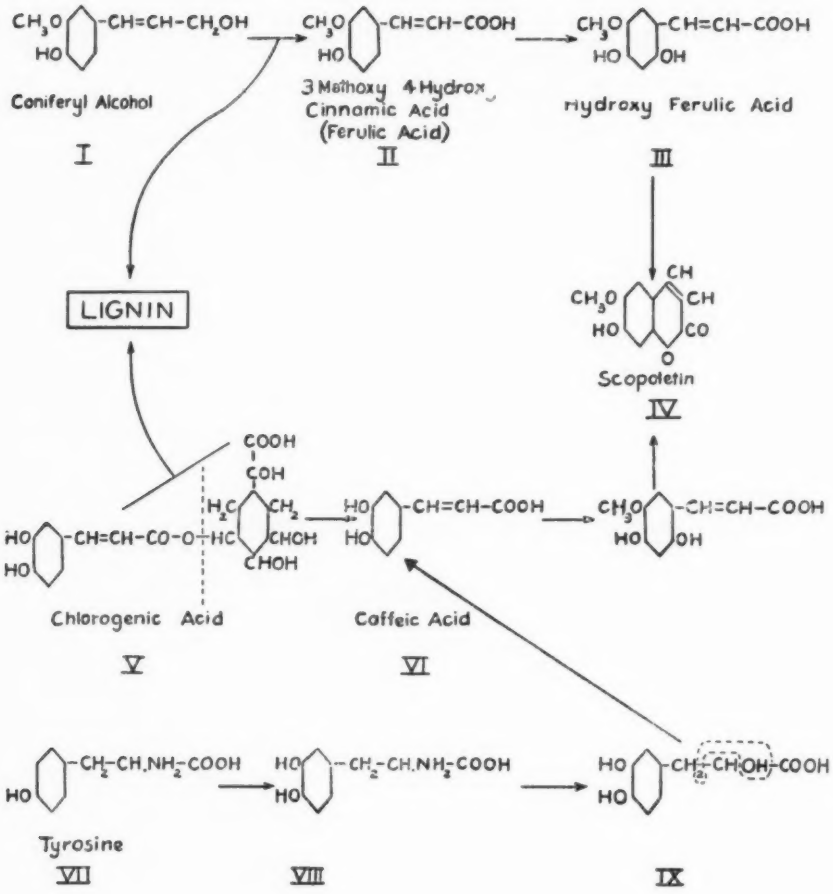


Fig. 3.

and the author discarded this idea at the outset in favour of the "broken chain" hypothesis, mainly because the greatest production of scopoletin in virus-infected leaves takes place in the mesophyll tissues from which lignin is considered to be absent. Lignin need not be brought into the discussion at all because coniferyl alcohol is present in any case, but since coniferyl alcohol is associated with lignin formation and because of the distribution of scopoletin in the plant and its very presence in the normal plant, the possible association of the two should not be overlooked.

The Decomposition of Tannin Hypothesis.

Wood (1933) has shown that in certain sclerophyll leaves lignin is produced at the expense of tannin. Here again the relationship may be through the phenyl-acrylic derivative, caffeic acid (VI). In any case, a splitting of the chlorogenic acid (V)—the tannin of tobacco leaves—to caffeic acid and the subsequent methylation and introduction of an (OH) group could yield scopoletin as an end-product. All these types of reaction may take place continually in plant systems.

The Proteolysis Hypothesis.

Another possibility has been suggested to the author by J. G. Wood, who pointed out the rôle which tyrosine (VII) might play. Tyrosinase could catalyse the production of the di-hydroxy compound (VIII) which could pass through (IX) to caffeic acid (VI).

Conclusion.

Many of the substances which take part in normal metabolic processes are in a state of dynamic equilibrium with each other. When cell injury causes a breakdown in organization these equilibria are upset and some substances may accumulate in abnormal amounts as a result. Furthermore, under these circumstances enzymes are often brought into contact with unusual substrates and abnormal chemical processes may proceed. There are, therefore, open to us a number of possible mechanisms to explain the formation of scopoletin in healthy plants and its accumulation in excessive amounts in virus-injured plants.

In the laboratory the lactone ring of scopoletin is surprisingly easily opened and the conversion of scopoletin to ferulic acid and its further reduction to coniferyl aldehyde or alcohol are all likely reactions. Alternatively, the production of scopoletin from any of its precursors by oxidation processes in health or disease as outlined earlier in this paper can be readily visualized. There is no reason why the excess scopoletin which appears as a result of virus activity should not be produced by a different path from the one by which it is produced in healthy plants, but simplicity requires that we assume the same mechanisms until we find that assumption to be inadequate.

While the question as to whether scopoletin is formed by a breakdown of lignin or other substances or as a result of the diversion of normal metabolism is still an open one, the author considers that on the meagre evidence available the latter is the more likely.

SUMMARY.

The distribution in healthy and virus-infected tobacco plants of the fluorescent 6-methoxy-7-hydroxy 1 : 2 benzo-pyrone (scopoletin) is described and some hypotheses in relation to its formation and possible part in metabolism are presented.

Scopoletin occurs in healthy tobacco plants from germination to maturity. The concentration falls off during the rapidly growing stage and reaches a maximum at maturity. Within the plant its greatest concentration is in the external endodermis followed by the internal endodermis. It is present in traces in the epidermis, hairs, cortex and pith. It is not normally observable in the xylem, cambium or phloem.

In tobacco plants infected with the virus of tomato spotted wilt, scopoletin occurs at greater concentrations although the relative distribution is as in healthy plants until the plant becomes saturated and all tissues fluoresce brightly.

The relation of scopoletin to lignin, chlorogenic acid (the tannin of tobacco leaves) and tyrosine is discussed from the point of view of its formation. It is suggested that the excess production of scopoletin in virus-infected plants is the result of a diversion of normal metabolism, rather than the direct results of a breakdown of lignin or other substance.

REFERENCES.

Andreae, W. A. (1947): Private Communication.
Best, R. J. (1936): Austral. J. exp. Biol., 14, p. 199.
Best, R. J. (1937): *Ibid.*, 15, p. 191.
Best, R. J. (1944): *Ibid.*, 22, p. 251.
Hibbert, H. (1942): Ann. Rev. Biochem., 11, p. 183.
Klein, G. (1932): Handbuch der pflanzenanalyse III/2, p. 845.
Wood, J. G. (1933): Austral. J. exp. Biol., 11, p. 139.

THE MICROBIOLOGICAL ASSAY OF FOLIC ACID

by MARGARET I. B. DICK, ISOBEL T. HARRISON AND K. T. H. FARRER

(From the Research Division, Kraft Walker Cheese Co. Pty. Ltd., Melbourne).

(Accepted for publication, 20th December, 1947.)

Over the past few years, methods of assay of folic acid and related compounds have been put forward by various workers. Until recently, however, when the structure of folic acid was determined, and its synthesis accomplished (Angier *et al.*, 1945) there was no absolute standard, and workers were using their own particular preparations as standards.

Snell and Peterson (1940) published a method of assay, using *L. casei* ε as test organism, and, as standard, a factor prepared from solubilized liver. Their basal medium contained optimum amounts of all the known requirements of the organism—acid hydrolysed casein, sodium acetate, glucose, tryptophane, cystine, riboflavin, niacin, calcium pantothenate, and mineral salts. Results were calculated from titration of the acid produced after incubation at 37° C. for two days.

Mitchell and Snell (1941) then used an assay method with crystalline Vitamin Bc (a Parke, Davis product) as standard, the basal medium being similar to that of Snell and Peterson, but with the addition of more B vitamins. Mitchell, Snell and Williams (1941) later modified the basal medium previously used by the addition of purines, and measured the response turbidimetrically.

In the same year, Williams (1941) published assay methods for some of the B vitamins, including folic acid. In his method for folic acid, he used his own preparation of folic acid as a standard. The basal medium contained hydrolysed casein, cystine, tryptophane, sodium acetate, glucose, purines, mineral salts, and six vitamins of the B complex—aneurin, riboflavin, pyridoxine, niacin, calcium pantothenate, and biotin. Response was measured turbidimetrically after sixteen hours' incubation at 30° C. The same author (Williams, 1942) then introduced another assay method, this time using *L. casei* ε with crystalline vitamin Bc as standard and, later, Burkholder *et al.* (1945) used essentially this method, with the addition to the basal medium of asparagine, glutamine, and p-aminobenzoic acid, the response being measured by titration of the acid produced after three days' incubation at 37° C.

Landy and Dicken (1942) published a general method of assay for six B vitamins, including folic acid, using *L. casei* ε and a folic acid preparation supplied by R. J. Williams. The basal medium included asparagine, and is given in detail later. After three days' incubation at 37·5° C., the acid produced was determined by titration.

Luckey *et al.* (1944) found that the method of Mitchell and Snell (*loc. cit.*) was unreliable when other than liver preparations were being assayed. They studied the effect of the various vitamins, purines, and salts on the organism, and subsequently modified the basal medium to give more consistent results. This medium is shown in Table 1.

Results were calculated from the response, measured turbidimetrically. The organism used was *S. lactis* R, and the standard was a series of duplicate tubes containing 0–300μg. of solubilized liver factor.

TABLE 1.

Basal Medium of Luckey, Briggs and Elvehjem.

Constituent.	Amt./litre.
Sodium acetate	4 gm.
Glucose	20 gm.
Casein, acid hydrolysed	10 gm.
l-cystine	200 mg.
l-tryptophane	600 mg.
Adenine sulphate	20 mg.
Guanine hydrochloride	20 mg.
Xanthine	20 mg.
Thiamine hydrochloride	0·4 mg.
Riboflavin	0·4 mg.
Nicotinic acid	1·2 mg.
Pyridoxine hydrochloride	2·4 mg.
Calcium pantothenate	0·8 mg.
Biotin (concentrate)	0·8 μg.
K_2HPO_4	10 gm.
Water to make	1 litre

Teply and Elvehjem (1945) then slightly modified the basal medium of Luckey *et al.* by the addition of buffers, so as to give maximum acid production with *S. lactis* R, and measured the response titrimetrically after incubation at 30° C. They made further modifications, including the addition to the basal medium of norit-treated peptone, to enable a satisfactory assay to be made with *L. casei* ε. They used crystalline Vitamin Bc as standard, and titrated the acid produced after 30–72 hours' incubation.

Preliminary experiments in this laboratory on the determination of folic acid using the naturally occurring substance as standard were not very satisfactory, but when synthetic pteroylglutamic acid became available it was decided to re-investigate the methods of assay available and to establish a satisfactory, reproducible assay method before proceeding to a study of the thermal stability of folic acid. This paper describes the work done.

EXPERIMENTAL.

Preparation of Standard Solution.

Synthetic pteroylglutamic acid (Folvite-Lederle Labs.) was used in all assays. The standard solution contained 5 μg./ml. of synthetic pteroylglutamic acid, prepared by dissolving 0·01 gm. folvite in glass-distilled water, with steaming, and making up to two litres. Recently, the folvite has been more easily dissolved in two litres of 50 p.c. alcohol solution as suggested by Barton-Wright (1947).

Preliminary work. Preliminary assays were tried out using the basal medium of Luckey, Briggs and Elvehjem, which has already been given. Both *L. casei* ε (A.T.C.C. No. 7469) and *S. faecalis* (A.T.C.C. No. 9790) were used.

Responses were read titrimetrically after incubation for 72 hours at 37° C. *L. casei* gave irregular results on this basal medium even when Speakman's salts (Speakman, 1923–4) were added as suggested by Luckey, Briggs and Elvehjem (1944). The standard curves obtained with *S. faecalis* were not consistent, irregular titration figures being obtained. This may have been a function of temperature.

It was decided to try the same two organisms using Landy and Dicken's basal medium (Table 2).

TABLE 2.

Basal Medium of Landy and Dicken.

Constituent.	Amt./litre.
Casein hydrolysate	10 gm.
Sodium acetate (hyd.)	12 gm.
Glucose	20 gm.
Asparagine	500 mg.
Tryptophane	200 mg.
Cystine	200 mg.
Salt solutions "A" and "B"	10 ml. ea.
Guanine hydrochloride	10 mg.
Adenine sulphate	10 mg.
Xanthine	10 mg.
Uracil	10 mg.
Thiamine hydrochloride	0·2 mg.
Biotin (free acid)	10 μg.
Riboflavin	0·4 mg.
Calcium pantothenate	0·4 mg.
Nicotinic Acid	0·4 mg.
Pyridoxine hydrochloride	0·8 mg.
Distilled water to	1 litre

Salt solution "A".		Salt solution "B".	
K_2HPO_4	5 gm.	$MgSO_4.7H_2O$	10 gm.
KH_2PO_4	5 gm.	NaCl	0·5 gm.
Water	50 ml.	$FeSO_4.7H_2O$	0·5 gm.
		$MnSO_4.2H_2O$	0·337 gm.
		Water	250 ml.

Again, however, *L. casei* ϵ gave very poor results (Fig. 1), with a low total acid production, but the response with *S. faecalis* was good, even at 37° C., and the blank was low, although the triplicates in the titrations were not good.

Assays were then carried out at 30° C. using *S. faecalis*, and the basal medium of Landy and Dicken (Fig. 2). Excellent triplicate titrations were obtained in all tubes, and the Landy and Dicken medium with *S. faecalis* gave such satisfactory results that it was decided to use this method as a standard procedure.

Later, the basal medium of Luckey, Briggs and Elvehjem was retested and found to be quite satisfactory on incubation at 30° C.

Preparation of inoculum. *S. faecalis* was stored in the refrigerator as a stab culture in yeast-whey agar,[1] with monthly transfer. To prepare the inoculum, cells from a stab culture were transferred to tubes of riboflavin basal medium (Kent-Jones *et al.*, 1946) and incubated at 30° C. overnight. The culture was then centrifuged, the cells resuspended in saline, and 1 ml. of this suspension diluted to about 20 ml. One drop of the final suspension per assay tube formed the inoculum.

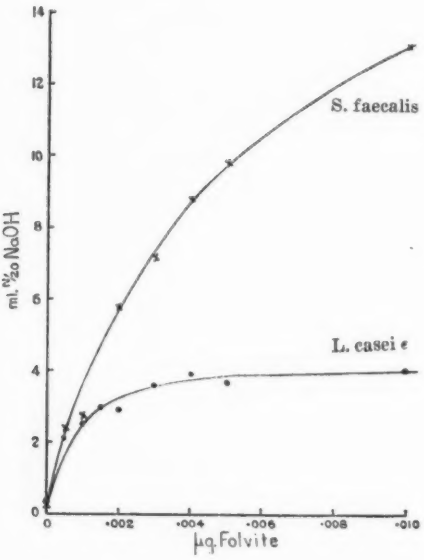


Fig. 1. Landy and Dicken Basal Medium.

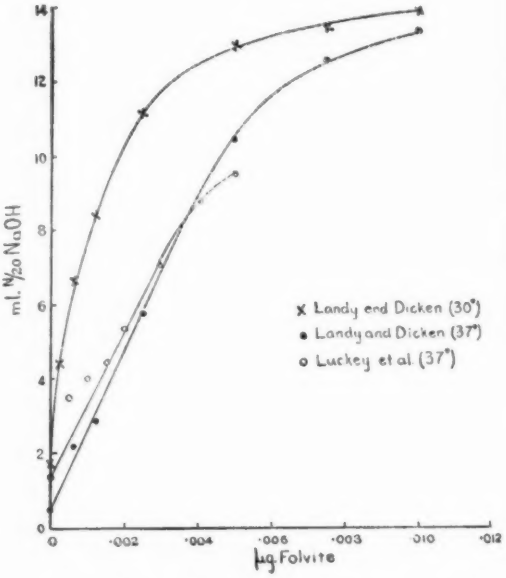


Fig. 2. *S. faecalis* Assay Curves on two Basal Media.

Method of assay. Ingredients for the basal medium were prepared by methods used by Barton-Wright (1944) in his niacin assay. However, instead of acid hydrolysed casein, trypsin-hydrolysed casein was used in some assays, with equal success. This was prepared as recommended by Kemmerer and Shapiro (1947).

After slight variations in the standard tube dosages, it was decided to use the following levels in triplicate: 0, ·000125, ·00025, ·000625, ·00125, ·0025, ·005 and ·0075 µg./tube. The tests were set up in duplicate at five levels, using 1, 2, 3, 4 and 5 ml. samples of test solution. 5 ml. aliquots of basal medium were added

[1] The ingredients of the yeast-whey agar used were: Whey powder, 4·5 p.c.; bacto-peptone (Difco), 0·5 p.c.; agar. 1·5 to 1·7 p.c.; yeast extract. 0·4 p.c.

to each tube, then the standard and test solutions were added, and the total volume per tube was finally made up to 10 ml. with glass-distilled water. The tubes were topped with aluminium caps, sterilized by autoclaving at 15 lb./15 min., cooled, and inoculated with a suspension of *S. faecalis*, prepared as above. After three days' incubation at 30° C., the acid produced was titrated with N/20 NaOH, using bromothymol blue as indicator.

It was thought at first that the use of aluminium caps, which have also been used by other workers, might have had an oligo-dynamic effect on the organisms, but comparison with cotton wool plugs showed that there was no difference between the two. It was found that these aluminium caps saved a great deal of time in the setting up of the assays.

Computation of results. Although good smooth standard curves were obtained in these assays, straight lines could not be drawn through the points satisfactorily, i.e. there was not a linear relation between ml. of NaOH used and concentration of folic acid (Wood, 1945). If, however, the log of the "response" was plotted against the log of the "dose" for the standards, as suggested by Wood (1947), it was found that a straight line could be drawn through the resulting points. Plotting a test solution in the same way, the points fell on a straight line which was parallel to the standard line. From these parallel lines the results were calculated according to Wood's method. This method in which the five different levels of assay were used to obtain the one result compared very well with the five results read directly from the standard curve.

DISCUSSION.

It would appear that two satisfactory media, differing mainly in the salts added and in the addition of asparagine to the Landy and Dicken medium, are available for the assay of folic acid. This fact, coupled with the availability of synthetic pteroylglutamic acid as a reliable reproducible standard has permitted the re-examination of the assay.

The Landy and Dicken basal medium used at 30° C. seems very satisfactory for assays of pteroylglutamic acid with *S. faecalis*, although the medium of Luckey, Briggs and Elvehjem also gave a good response with the same organism under the same conditions. The inconsistent results obtained with this medium at 37° C. were probably caused by the higher temperature of incubation.

L. casei ε does not appear to be a satisfactory organism for the assay of pteroylglutamic acid, as nothing like maximum acid production could be obtained on the basal media studied.

The samples assayed to date have been synthetic pteroylglutamic acid solutions, subjected to various physical treatments. Hence, there are no interfering substances present in the test solutions to affect the growth of the organism, and the growth response is directly proportional to the added material.

The Wood log-log method of calculation appears to be most satisfactory. The standard points were found to fall readily along a straight line, with the exception of the highest points, where acid production had reached a maximum. The example shown (Fig. 3) is the same curve as is plotted to show response to the Landy and Dicken medium at 30° C. (Fig. 2), and it can be seen that the points fall much closer to a straight line in Fig. 3. The results agree well with those calculated by direct reading from the standard curve, but in the log-log method the reliability of the assay is easily assessed from the degree of parallelism between the standard and test lines.

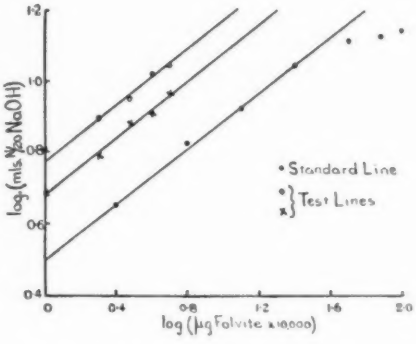


Fig. 3. Log-Log Method of Calculation.

No attempt has yet been made to apply these modified conditions to the determination of folic acid in foodstuffs, as the availability of the various pteroylglutamic acids to the organism is still in doubt. The whole question of the enzymic hydrolysis of the conjugates requires further exploration.

SUMMARY.

A modification of the Landy and Dicken method of assaying pteroylglutamic acid (synthetic "folic" acid), by using *Streptococcus faecalis* as assay organism, has been presented.

The Wood log-log relationship method of calculation of results has been tried and found successful for these particular assays.

Acknowledgment. The authors wish to thank the Directors of the Kraft Walker Cheese Co. Pty. Ltd. for permission to publish this paper.

REFERENCES.

Angier, R. B. *et al.* (1945): Science 102, p. 227.
Barton-Wright, E. C. (1944): Biochem. J., 38, p. 314.
Barton-Wright, E. C. (1947): "Practical Methods for the Micro-biological Assay of the Vitamin B Complex and Essential Amino Acids." Ashe Laboratories Ltd.
Burkholder, P. R., McVeigh, I. and Wilson, K. (1945): Arch. Biochem., 7. p. 287.
Kent-Jones, D. W. and Barton-Wright, E. C. (1946): Analyst, 71, p. 397.
Kemmerer, A. R. and Shapiro, F. (1947): Anal. Chem., 19, 5. p. 358.
Landy, M. and Dicken, D. M. (1942): J. lab. clin. Med., 27, p. 1,086.
Luckey, T. D., Briggs, G. M. and Elvehjem, C. A. (1944): J. biol. Chem., 152, p. 157.
Mitchell, H. K. and Snell, E. E. (1941): Proc. Nat. Acad. Sci., 27, p. 1.
Mitchell, H. K., Snell, E. E. and Williams, R. J. (1941): J. Amer. chem. Soc., 63, p. 2,284.
Snell, E. E. and Peterson, W. H. (1940): J. Bact., 39, p. 273.
Speakman, H. B. (1923-4): J. biol. Chem., 58. p. 395.
Teply, L. J. and Elvehjem, C. A. (1945): *Ibid.*, 157, p. 303.
Williams, R. J. (1941): Univ. Texas Publn., 4,175, p. 7.
Williams, R. J. (1942): *Ibid.*, 4,237.
Wood, E. C. (1945): Nature, 155, p. 632.
Wood, E. C. (1947): Analyst, 72, p. 84.

THE THERMAL STABILITY OF FOLIC (*PTEROYLGLUTAMIC*) ACID

by MARGARET I. B. DICK, ISOBEL T. HARRISON AND K. T. H. FARRER

(From the Research Division, Kraft Walker Cheese Co. Pty. Ltd., Melbourne).

(Accepted for publication, 20th December, 1947.)

The successful degradation and synthesis of the accessory food factor variously known as vitamin Bc, *L. casei* factor, *S. lactis* R factor, folic acid and, finally, pteroylglutamic acid (Angier *et al.*, 1946) has not only given rise to more exact work on the significance of this vitamin in anaemia of different kinds, but has also enabled a more precise standardization of the microbiological assay of the factor to be made. Such assay, of course, involves the heat sterilization of the standard test solutions containing folic acid and, as little is known of the thermal stability of this vitamin, this study was undertaken. The first step was to establish the relation between pH and recovery of added folic acid at temperatures of 100° and 121° C. (15 lb. steam pressure), concurrently investigating the effect of various buffer solutions. The second step was to study the kinetics of the thermal destruction demonstrated.

EXPERIMENTAL.

MATERIALS.

Folic acid stock solution. Synthetic pteroylglutamic acid ("Folvite" of Lederle Laboratories) was used. 10 mg. were dissolved in distilled water by steaming and made up to 2 litres. Later a 50 p.c. water-alcohol mixture was used to dissolve the "Folvite". 1 ml. of stock solution contained 5μg. of "Folvite".

Buffer Solutions.

(a) *Sodium Acetate-Hydrochloric Acid.* A 1 M solution of sodium acetate was prepared in distilled water. For most experiments 10 ml. were diluted to about 40 ml. and the pH adjusted to the desired value with 6 N HCl. The volume was then made up to 50 ml. In preparing larger volumes 50 ml. of acetate after pH adjustment were made up to 250 ml.

(b) *Sodium Phosphate-Citric Acid.* Solutions of 0·1 M citric acid and 0·2 M disodium hydrogen phosphate were prepared. Varying volumes of the one were made up to 50 ml. with the other to yield the desired pH. Any final adjustment was made with 6 N HCl or 10 p.c. NaOH.

(c) *Phosphate-Phthalate.* 10 ml. of a solution 0·5 M with respect to both potassium dihydrogen phosphate and potassium hydrogen phthalate were diluted to about 45 ml., the pH adjusted to the desired value with NaOH solution and the volume made up to 50 ml.

(d) *Glycine-Sodium Hydroxide.* A solution of glycine (0·1 M) was prepared in 0·1 N NaCl solution and varying volumes as required were made up to 50 ml. with 0·1 N NaOH.

EXPERIMENTAL PROCEDURE.

(a) *Effect of pH at 100° C.* 50 ml. of buffer solution and 5 ml. of folic acid stock solution in a 150 ml. CO_2 flask were connected to a reflux condenser by means of a ground glass joint. As folic acid is light sensitive, the flask was surrounded by a metal air bath fitted with a lid. The solution was boiled over a Bunsen flame and boiling continued for exactly one hour when cold water was poured down the condenser to quench the test solution and the volume rapidly made up to 500 ml. in a volumetric flask. This solution representing a dilution of 1–100 of the original folic acid solution was used for the assay. Separate tests established that shift in pH was negligible. All pH values are correct to ± 0·04 pH units and were measured with a glass electrode.

(b) *Effect of pH at 121° C.* 50 ml. of buffer solution and 5 ml. of folic acid stock solution were added to 250 ml. conical flasks which were plugged with cotton-wool (protected by tin foil). The flasks were autoclaved at 15 lb. (121° C.) for 15 minutes (exclusive of 10 minutes heating and 10 minutes cooling) which is the treatment given to actual assay tubes. After removal from the autoclave the solutions were made up to 500 ml. with cold water and assayed.

(c) *Reaction velocity curves at 100° C.* 100 ml. of sodium acetate-HCl buffer solution of the desired pH were made up to include 10 ml. of folic acid stock solution and boiled under reflux away from the light. At regular time intervals (the exact time varying with the pH of the solution) samples were withdrawn from the solution by means of a pipette and cooled rapidly. Exactly 10 ml. were then pipetted into a 100 ml. volumetric flask and made up to volume. This solution representing a 1–100 dilution of the original folic acid stock solution, was assayed. The pH of the unused solution was checked.

(d) *Reaction velocity curves at 120° C.* Autoclaving the solutions was far too cumbersome a method for samples to be taken at regular time intervals. Apart from this, while it is one thing to compare solutions under the same conditions as those experienced by assay tubes, it is another to attempt to carry out kinetic studies with heating and cooling periods of 10 minutes each wherein destruction is also likely to occur. Accordingly, sealed ampoules were used in an oil bath at 120° C. 100 ml. of sodium acetate-HCl buffer of the desired pH were made up to include 10 ml. of the folic acid stock solution and exactly 10 ml. were pipetted into each of six 10 ml. glass ampoules which were then sealed. The ampoules were placed on a gauze tray immersed in an oil bath thermostatically controlled at 120 ± 1° C. The oil was of such a depth that about half an inch of the long necks of the ampoules was visible above the oil surface when the tubes stood vertically. The bottom of the gauze tray was raised one inch from the bottom of the oil bath so that, with mechanical agitation there was complete circulation of oil round the ampoules. A preliminary trial with a typical ampoule open, and containing a thermometer, showed (by extrapolation) that the temperature of the solution would rise to 120° C. from atmospheric temperature in about 5½ minutes. When, therefore, a batch of ampoules was placed in the bath the first was removed after 6 minutes and the folic acid therein was taken as "a" in the unimolecular reaction equation. (The difference between the amount of folic acid in this solution and that in a control was a measure of the amount destroyed as the solution was heating to 120° C. This was significant at pH 1 and 2 and justified the adoption of the preliminary 6 minute heating period). The remaining ampoules were then removed at the desired time intervals, for pH 1 every 3 minutes, for pH 2 every 10 minutes and for pH 3 every 30 minutes. As soon as they were removed from the oil bath the ampoules were agitated vigorously in a bath of water in equilibrium with ice and fell to atmospheric temperature within 1 minute. The contents of each ampoule were then

made up to 100 ml. and assayed. A control ampoule was retained in the bath throughout the full heating period and, on cooling, was used to check any pH drift. No drift was found in any of these solutions.

Method of assay. The solution under test, already at a dilution of 1 in 100 was further diluted to give a solution of approximately ·0005μg. of folic acid/ml. 1, 2, 3, 4 and 5 ml. samples were used in the standard assay method, which has been described elsewhere (Dick, Harrison and Farrer, 1948).

RESULTS.

The percentage recovery of folic acid added to various buffer solutions of different pH values is tabulated in Table 1 (100° C.) and Table 2 (121° C.), and the average recovery for all buffers at each pH is plotted in the graphs (Figs. 1 and 2).

TABLE 1.

P.c. recovery of folic acid from buffer solutions after 1 hour at 100° C.

pH.	Sodium acetate-HCl.	Sodium phosphate-citric acid.	Phosphate-phthalate.	Glycine-sodium hydroxide.
1	54, 66, 41, 39			
2	80, 64, 86	72, 82, 85		
3	68, 88, 90	71, 85, 88, 101		
4	97, 87	95, 84, 92		
5	92	95, 91, 87	89, 100	
6		106, 103, 100	100, 103	
7		106, 95	104, 100	
8		95, 100, 96		100, 96, 100
9				96, 100, 100
10				108, 103, 100

TABLE 2.

P.c. recovery of folic acid from buffer solutions after autoclaving 15 mins. at 15 lb. pressure (121° C.).

pH.	Sodium acetate-HCl.	Sodium phosphate-citric acid.	Glycine-sodium hydroxide.
1	29, 32		
2	66, 67		
3	80, 90	98, 90	
4	96	96	
5	100	100	
6		93, 100, 100	
7		100	
8			100
9			100
10			100

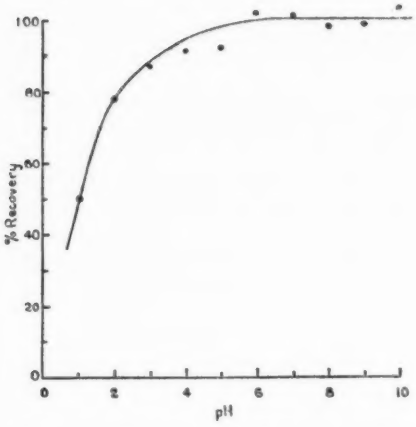


Fig. 1. Recovery of added folic acid on boiling 1 hour at various pH.

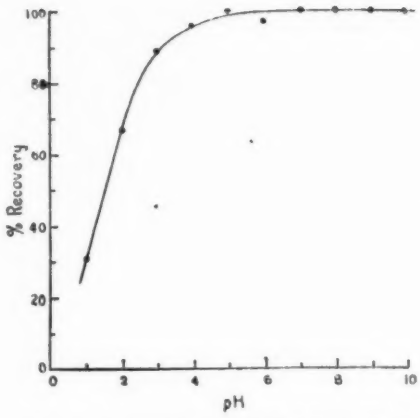


Fig. 2. Recovery of added folic acid on autoclaving 15 mins. at 15 lb. (121° C.) at various pH.

The reaction velocity, k, for a unimolecular reaction is calculated from the equation $k = \frac{2{\cdot}303}{t} \log \frac{a}{a-x}$, where a is the original concentration of the substance and x is the amount destroyed in time t (measured in minutes). That a reaction is unimolecular is shown by the linearity of the curve obtained by plotting log $(a-x)$ against t. The curves shown in Fig. 3 are typical of those obtained with folic acid. That the thermal destruction of folic acid is a unimolecular reaction is amply demonstrated (Fig. 3) and the reaction velocity, k, for pH 1, 2 and 3 has been calculated at both 100° C. and 120° C. In each case, this was done by plotting log $(a-x)$ against time and calculating k from the slope of the curve rather than by direct calculations with actual values of $(a-x)$. This procedure, by using a best straight line, averages a series of results obtained by means of a relatively inexact analytical method and has been used successfully by one of the authors (K.T.H.F.) in a series of studies on aneurin and cocarboxylase. The very rapid abnormal rate of destruction in the early part of some experiments (for an example of this see Fig. 3) has also been encountered in studies with aneurin. There appears to be no satisfactory explanation for it, nor is it a constant phenomenon. However, in every case encountered (with both aneurin and folic acid) the reaction velocity quickly settles down to the true steady value.

The values of k obtained are tabulated in Table 3.

TABLE 3.

Velocity coefficient for thermal destruction of folic acid.

pH.	k 100° C.	k 120° C.
1	·0070 ·0078 ·0067	·052 ·053 ·043
2	·0020 ·0023 ·0029	·017 ·018 ·012
3	·0014 ·0010	·0065 ·0065

DISCUSSION.

In the first place it is quite apparent that low pH values favour the thermal destruction of folic acid. It is also very apparent that as the pH rises the vulnerability of folic acid rapidly decreases, until at pH 5 one may regard it as stable to heat. As one would expect, the higher temperature of 121° C., even for a much shorter time interval, serves to magnify the vulnerability of folic acid at the lower pH values without at all affecting the overall picture of thermal destruction. This follows from Figs. 1 and 2 and is confirmed quantitatively by the figures presented in Table 3. Even though only one buffer solution was used

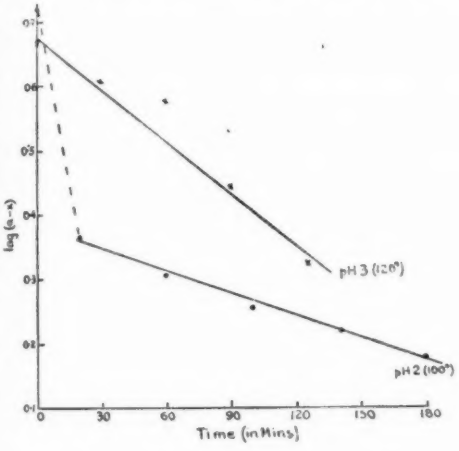


Fig. 3. Log $(a - x)/t$ for the thermal destruction of folic acid.

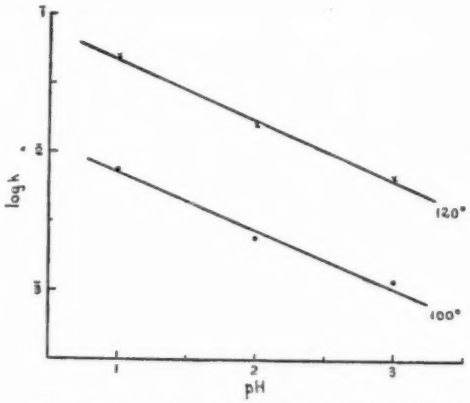


Fig. 4. Log k/pH. for destruction of folic acid at 100° and 120° C.

for the reaction velocity studies, it is evident from recoveries from different buffer solutions at different pH values that the variation in thermal destruction is a function of pH only and does not depend also on the constituents of the buffer solution as does the thermal destruction of aneurin (Beadle, Greenwood and Kraybill, 1943; Farrer, 1945). There is a substantial deviation from the mean in each group of figures, but no more so (except for pH 1 and 2 at 100° C.) than would be expected from a microbiological assay procedure wherein one must be prepared for errors of ±10 p.c. Fig. 2 is a much smoother curve than Fig. 1, but as it confirms what is to be expected from the results obtained at 100° C., the number of experiments carried out was much smaller than at 100° C.

In the case of pH 1 and 2 at 100° C. there are deviations of +32 p.c. and −22 p.c. at pH 1 and +10 p.c. and −18 p.c. at pH 2 respectively. These are too large to be ascribed to errors in the assay but it is quite likely that they are due to variations in the initial abnormal rate of destruction which, as already pointed

out, is not constant. Indirect confirmation of this may be found in the three reaction velocity experiments carried out at 100° C. and pH 1. The values found for k were ·0070, ·0078, ·0067, but the corresponding percentage recoveries after 1 hour were 68, 54 and 48.

The reproducibility of the values for k is quite good in view of the limitations of the assay method, and was best at pH 3 where the time intervals used in the experiment were longer. It was natural to look for some quantitative relation between k and pH, and log k was plotted against pH (Fig. 4). As pointed out in another connection (Farrer, 1945), there is evidence to support the contention that changes in dissociation between 20° C. and 100° C. are not large. In any case, k at 100° C. or 120° C. may still be related to hydrogen ion concentration at 20° C.

Two parallel straight lines resulted, indicating two things:

(i) k, the velocity coefficient for the thermal destruction of folic acid, is directly proportional to the hydrogen ion concentration of the solution.

(ii) As would be expected, an increase in temperature increases the value of k without interfering with the relation between k and the hydrogen ion concentration. This would follow from the Arrhenius equation which relates k to the absolute temperature, all other conditions being constant.

So far as the original question which prompted this work is concerned, it is safe to say that no loss of folic acid will occur in sterilizing media at the usual pH of about 6·8.

SUMMARY.

The thermal stability of synthetic folic (pteroylglutamic) acid has been studied at 100° C. and 120±1° C. by means of the microbiological assay, using *S. faecalis* as the test organism.

Considerable destruction may be expected at pH values below 4, but above pH 5 there is no destruction in 1 hour at 100° C. or 15 minutes at 121° C. Different buffer salts appear to be without effect.

The thermal destruction is a unimolecular reaction and values of k, the velocity coefficient, at pH 1, 2 and 3 at both 100° C. and 120° C. have been determined. The linearity of the log k/pH curves shows that k is directly proportional to the hydrogen ion concentration and increases in temperature increase k by the same amount at any given pH.

Acknowledgment. The authors wish to thank the Directors of the Kraft Walker Cheese Co. Pty. Ltd., for permission to publish this paper.

REFERENCES.

Angier, R. B. *et al.* (1946): Science, 103, p. 667.
Beadle, B. W., Greenwood, D. A. and Kraybill, H. R. (1943): J. biol. Chem., 149, p. 339.
Dick, M. I. B., Harrison, I. T. and Farrer. K. T. H. (1948): Austral. J. exp. Biol., 26, p. 231.
Farrer, K. T. H. (1945): Biochem. J., 39, p. 128.

LETHAL MUTATIONS PRODUCED BY ULTRA-VIOLET AND X-RAY IRRADIATION OF FUNGAL SPORES

by JOAN MUNRO FORD

(From the Physics Department, University of Tasmania).

(Accepted for publication, 23rd December, 1947.)

When fungal spores of *Chaetomium globosum* are irradiated with ultra-violet light or X-rays, many of the spores germinate when plated on malt agar, but the young colonies stop growing and die when less than a millimetre in diameter. These colonies have well marked characteristics and can be recognized as identical with others that appear through many generations in the progeny of known mutations. Because of their microscopic size when growth ceases they have been called "microscopic mutants." They do not occur in the progeny of normal colonies.

The microscopic mutants have been studied especially in a mutation which is produced selectively by short ultra-violet irradiation of fungal spores, and

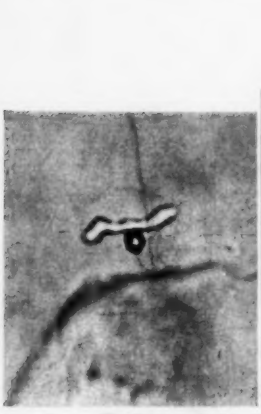


Fig. 1.

Fig. 2.

Fig. 3.

EXPLANATION OF FIGURES.

Figs. 1–6. Photomicrographs of colonies of *Chaetomium globosum* Fld grown from single spores plated on malt agar. All unstained. Figs. 1–5 show microscopic mutants grown from irradiated spores 50 hours or more after spore plating, and Fig. 6 shows a normal colony from an unirradiated spore about 35 hours after spore plating.

Fig. 1. Small microscopic mutant showing spore and one hypha. × about 500.

Fig. 2. Microscopic mutant showing close branching (A), and deflated appearance (B) of the few hyphae. × about 250.

Fig. 3. Microscopic mutant showing one straggly unbranched hypha. × about 250.

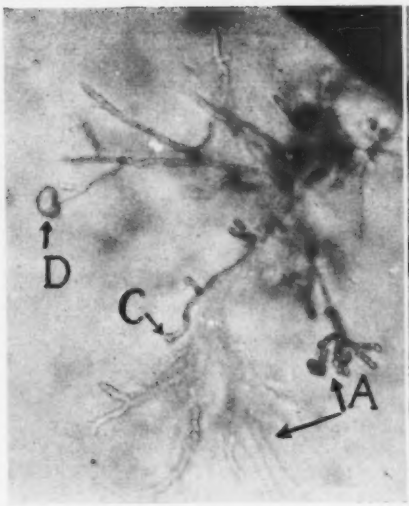


Fig. 4.

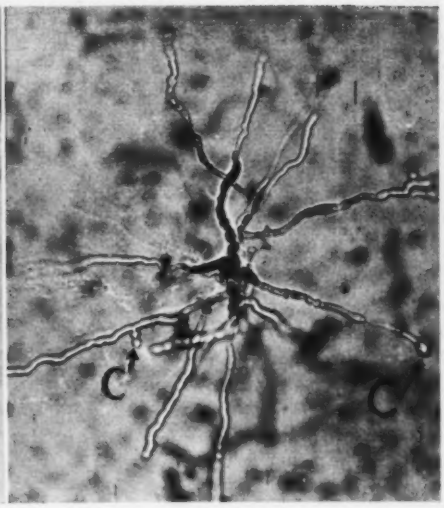


Fig. 5.

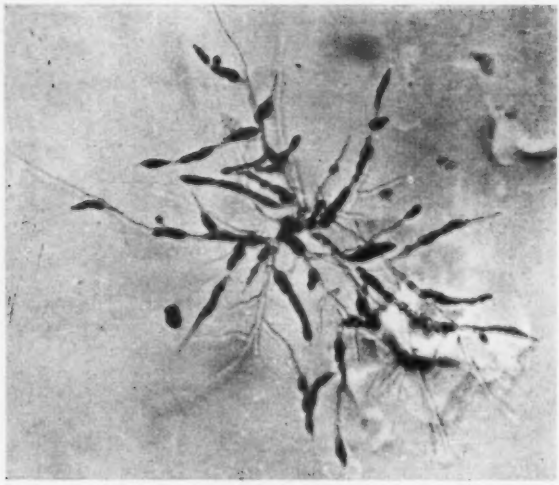


Fig. 6.

Fig. 4. Microscopic mutant showing features of close branching (A), swollen hyphal tips (C), and extruded contents (D) of some hyphae. × about 300.

Fig. 5. Microscopic mutant with swollen hyphal tips (C). × about 350.

Fig. 6. Colony grown from a normal unirradiated spore. Branching and growth normal. × about 300.

appear in all generations examined. This mutation has been described by Ford (1946). They have also been found to accompany another mutation which has been produced by X-rays.

Experiments have been made on the production of mutations by ultra-violet from wavelengths 2482 A.U. to 3656 A.U., and even with a visible violet line 4047 A.U. A number of the experiments are described in previous papers (1945, 1946, 1947). Three thousand one hundred and nineteen (3,119) microscopic mutants in five thousand six hundred and eighty-two (5,682) germinated spores, plated singly, have been obtained in these experiments. Large numbers have also been obtained in X-ray experiments. The percentages of microscopic mutants produced have been a useful check on the dose of irradiation given, as the higher the dose of radiation the greater the percentage of microscopic mutants. Less than 1 p.c. occurred in control colonies.

EXPERIMENTAL.

Description and Growth Characteristics of Microscopic Mutants.

As reported in previous papers (1945, 1946 and 1947) the material used was the fungus *Chaetomium globosum* Fld., which is itself a saltant produced in irradiation experiments Spores of this fungus, after irradiation, are plated singly on cleared 2 p.c. malt agar and examined daily for germination and microscopic mutants. The spores are $8\mu \times 10\mu$ in diameter and plating and examination is done with the aid of a dissecting microscope (1945). Germination of the normal spores takes place 6–8 hours after plating and microscopic mutants are apparent about 30 hours from plating.

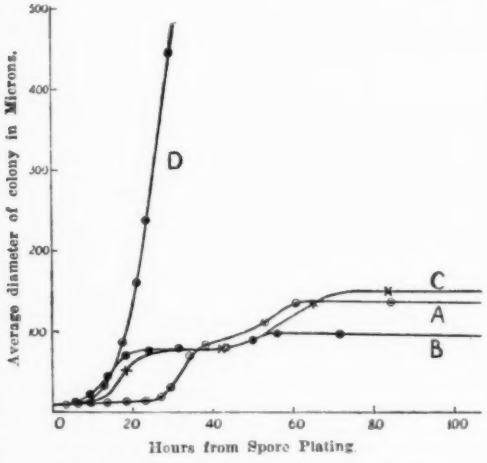


Fig. 7. Graph of the average diameter in microns of microscopic mutant colonies A, B and C, and normal colony (D) of *Chaetomium globosum* Fld, plotted against time in hours from plating of the spores (singly). A, represents a mutant from a spore irradiated by the short ultra-violet wavelength, 2804 A.U.; B, represents a microscopic mutant from a spore irradiated by X-rays, and C, represents a microscopic mutant from the progeny of a K spore mutation.

Figs. 1–5 show examples of microscopic mutants. These photos were taken 50 hours at least after plating of the spores, when growth of the colony had ceased. The normal colony at 50 hours after spore plating is about 6 mm. in diameter. Fig. 6 shows a normal colony about 35 hours after spore plating.

The microscopic mutants are small; they may cease growing when the germ tube has just protruded and is little more than the length of the spore (Fig. 1), or they may continue to grow until almost a mm. in diameter (Figs. 4 and 5). The majority lie between these two extremes of size (Figs. 2

and 3). They are characterized by this early cessation of growth; by abnormal branching, sometimes closely branched (Figs. 2 and 4) and sometimes sparsely branched (Fig. 3). Another feature which is common to most of these colonies is the bursting of some of the hyphal tips, with a subsequent disintegrated, deflated, appearance of the hyphae. Fig. 4 shows protoplasm extruded from the ends of some of the hyphae, and Fig. 2 shows deflated hyphae. Fig. 5 shows swollen hyphal tips, just before bursting.

Fig. 7, A, B and C (and see Ford, 1946[1]), shows the rate of growth of some microscopic mutants. Measurements were made of the diameter with an eyepiece micrometer in two directions at right angles to each other. Examples have been chosen from colonies produced in the following way: (A) by direct irradiation with 2804 A.U., a short ultra-violet line, and (B) by X-irradiation. (C) refers to microscopic mutants from the progeny of the selectively produced K mutation, and (D) refers to normal colonies. It will be seen that A, B and C, in contrast to D, show short periods of normal growth alternating with periods when growth is very slow or has ceased altogether. Growth takes place over a period of 10–70 hours. After 70 hours, growth has usually ceased and bursting of hyphal tips is common. A, B and C all show this type of growth which is similar to the growth of the K mutation (Ford, 1946). Some colonies do not show more than one growth period. Examples of both types of growth may appear in microscopic mutants produced directly by ultra-violet or X-irradiation as well as in microscopic mutants appearing in the progeny of mutations.

Association with Macroscopic Mutations.

Attempts have been made to grow the microscopic mutants until they produce spores and to observe what happens to subsequent generations. It was thought that they might be due to deficiencies and, therefore, similar to the biochemical mutants of *Neurospora*. (Beadle, 1945).

Small quantities of the vitamins A, B_1, B_2, C and D, also peptone and yeast have been added to the media on which the spores were plated. Different media, for example oatmeal agar, and prune agar have been used for plating the spores. No appreciable improvement in the growth has been obtained, and further experiments are required on this point.

Some young colonies which grew slowly at first continued to grow and became mutations with known characters. These, themselves, may produce progeny with a high percentage of microscopic mutants. In the K mutation eleven generations of spores have been examined and microscopic mutants obtained from all generations. They are, therefore, hereditable in that they occur in many generations associated with mutations.

Table 1 shows the number of germinated spores plated singly (column c), the number of microscopic mutants (column d), and the percentage of microscopic mutants in the total number of germinated spores (column e), for 5 of the K mutations, a mutant produced by X-rays, and a group of 4 mutants, 2 of X-ray origin, and 2 of ultra-violet origin.

Remembering that 0·6 p.c. are produced in normal colonies of *Chaetomium globosum*, the percentages of microscopic mutants in the progeny of the mutations tabulated above are very high. The K mutations produce an average of 32 p.c. of microscopic mutants in their progeny, and the X-ray mutant 71 p.c.

The types of microscopic mutants associated with the mutations are similar to those produced directly by ultra-violet and X-radiation (Fig. 7). There is some difference between the ultimate size and form in the K mutation progeny and the X-ray mutation progeny. Both have the very small microscopic mutants which are less than, or barely three times the length of the spores (Fig. 1), but associated with the K mutant are many closely-branched microscopic mutants between 100μ and 400μ in average diameter which are not characteristic of the

[1] In Ford (1946) the microscopic mutants were called "non-visible" colonies.

X-ray mutant. Associated with the X-ray mutant about half the lethals are just less than a millimeter in diameter, for example Fig. 5, but this type is not characteristic of the K mutation progeny.

TABLE 1.

Numbers and percentages of microscopic mutations occurring in germinated spores, plated singly, of the progeny of macroscopic mutations produced by ultra-violet light or X-rays.

(a). Mutant.	(b). Number of generations.	(c). Number of germinated spores.	(d). Number of microscopic mutants.	(e). Microscopic mutants in germinated single spores.
K spore mutants		singly plated		per cent.
Ka	11	640	177	28
Kb	5	80	13	16
Kh	4	112	34	30
Ki	4	40	22	55
Kj	3	77	25	32
X-ray mutant	1	158	113	71
4 others, 2 produced by u.v. and 2 by X-rays.	1	34	5	15
Normals	many	3,024	20	0·6

Production of Microscopic Mutants by Direct Irradiation with Ultra-violet Light and X-rays.

Many experiments have been made on the production of macroscopic mutations and microscopic mutants by ultra-violet irradiation and X-irradiation of the fungal spores (Ford, 1945, 1946, 1947). It was found that the percentage of microscopic mutants was closely associated with the dose given at a particular wavelength. As figures for microscopic mutants were obtained much more easily than could be done with macroscopic mutants, the percentage microscopic mutants was used as a biological measurement to check doses required to produce them at different wavelengths.

For the doses of irradiation used, the percentage germination was similar to that of the controls, but the percentage of microscopic mutants increased with increasing dose at each wavelength. It was not until almost a hundred per cent. microscopic mutants were obtained that the percentage germination of the spores was affected at the short ultra-violet lines thoroughly investigated.

Table 2 gives the number of germinated spores (column d), the numbers of microscopic mutants at different wavelengths of ultra-violet light from 2482 A.U. to 3656 A.U., the violet line 4047 A.U., and X-rays (column e). The average doses required to produce them has been given for the ultra-violet lines in column b, and the percentages of microscopic mutants produced in the germinated spores plated singly is shown in column e.

TABLE 2.

Numbers and percentages of microscopic mutants in germinated spores, plated singly, produced by wavelengths of ultra-violet and violet light, and X-rays.

(a). Experimental.	(b). Average dose in Joules/cm.^{2}.	(c). Number of microscopic mutations.	(d). Number of germinated spores.	(e). Percentage microscopic mutants in germinated single spores.
			single spores	per cent.
Controls	—	20	3,024	0·6
2482 A.U.	1·44	278	382	73
2654 A.U.	0·43	564	1,008	56
2804 A.U.	0·2	391	614	63
2967 A.U.	0·71	127	273	46
3025 A.U.	2·9	458	1,305	35
3132 A.U.	26·6	973	1,237	77
3342 A.U.	150	163	410	40
3656 A.U.	900	135	315	43
4047 A.U.	1,800	30	138	22
Totals Rows 2–10	—	3,119	5,682	54·0
X-rays	—	741	1,371	54·0
Totals Rows 2–11	—	3,860	7,053	54·0

Table 2 gives an indication of the numbers of microscopic mutants which have been obtained over a number of years in many experiments on the irradiation of fungal spores. The percentages are an average for many experiments, as is the dose when given. Actually the higher the dose at each wavelength the greater the percentage of microscopic mutants produced. If the right dose is given any percentage of microscopic mutants between 0 and 100 should be obtainable. A much higher dose than shown in Table 2 is required to prevent germination of all spores irradiated.

Measurements made on microscopic mutants produced by irradiation of the spores at increasing doses of the short ultra-violet line 2804 A.U., showed that on the whole the higher the percentage of microscopic mutants produced the smaller the average size of the colonies when growth ceased.

SUMMARY.

When spores of *Chaetomium globosum* (Ascomycetes, Sphaeriales) are irradiated with monochromatic ultra-violet and violet light and with X-rays, many small lethal colonies are obtained. They do not occur normally. The small lethal colonies are also found associated with macroscopic mutations of known characteristics and appear in their progeny for many generations. As they are hereditable in this way it has been thought justifiable to call them mutations, and the term "microscopic mutants" has been used for them throughout this paper. The term microscopic is used because they are less than a millimetre in diameter when growth ceases. This designation distinguishes them from the "macroscopic mutations" which have distinct features visible to the naked eye different from those of normal colonies. The microscopic mutants and their associations with macroscopic mutations have been described. A total of 389 of the former were produced in a total of 1,141 germinated spores of mutation progeny.

Their direct production by ultra-violet, violet and X-irradiation is also recorded and tabulated. A total number of 3,860 microscopic mutants in 7,053 germinated spores were produced.

Acknowledgments. Sincere thanks are expressed to Professor McAulay for helpful assistance during the preparation of this paper. The funds for the work came from the Commonwealth Grant to the University.

REFERENCES.

Beadle, G. W. (1945): Physiol. Rev. 25, p. 643.
Ford, J. M. (1946): Austral. J. exp. Biol., 24, p. 241.
Ford, J. M. (1947): J. gen. Physiol., 30, p. 211.
McAulay, A. L. and Ford, J. M. (1947): Heredity, 1, p. 247.
McAulay, A. L., Plomley, N. J. B. and Ford, J. M. (1945): Austral. J. exp. Biol., 23, p. 53.

RHYTHMIC CONTRACTIONS OF URETER

by W. T. AGAR

(From the Department of Physiology, Melbourne University).

(Accepted for publication, 2nd January, 1948.)

The few studies that have been made on the properties of the muscle of the ureter indicate that this tissue characteristically responds to stimulation with a series of rhythmic contractions.

Using pig's ureter, Gruber (1928, 1930) observed such contractions to occur both spontaneously and under the influence of adrenaline. Burgi (1945) observed that adrenaline induced rhythmic contractions in the guinea-pig's ureter. Stoicesco (1929) cannulated the isolated ureter of the dog and noted that distension induced rhythmic contractions whose frequency varied, within limits, with the internal pressure.

Bozler (1938), in a study of electrical stimulation of smooth muscle, noted the long refractory period of rabbit and guinea-pig ureter. He also noted the all-or-none behaviour of ureter and considered that the muscle of the ureter formed a syncytium.

These properties of rhythmicity and long refractory period recall the properties of heart muscle. Further similarities between cardiac and ureteric muscle became evident in the following experiments.

METHOD.

Guinea-pigs were used in 25 experiments; in a few others cats and rabbits were used. The animal was stunned by a blow on the head and bled out. The ureters were removed and placed in Ringer-Locke solution of the following composition: NaCl 0·9 gm., KCl 0·042 gm., $CaCl_2$ 0·024 gm., $MgCl_2$ 0·00025 gm., $NaHCO_3$ 0·05 gm., dextrose 0·05 gm., distilled water to 100 ml. In some experiments 2 or 3 cm. of ureter were suspended in an organ bath of 15 ml. capacity containing oxygenated Ringer-Locke solution at 37·5° C., and attached to a frontal writing lever exerting a tension on the ureter of 0·5 gm. weight. In other experiments a cannula was inserted into the renal end of the piece of ureter; the cannula was connected to a reservoir of Ringer-Locke solution which could be raised or lowered, thus altering the hydrostatic pressure within the ureter. The ureter was suspended in the organ bath, the cannula forming the lower fixed attachment. The free end of the ureter was transfixed by a hook connected to the writing lever.

The effects of adding various substances to the Ringer-Locke solution surrounding the ureter, and of different internal hydrostatic pressures in the cannulated ureter, were studied.

RESULTS.

Experiments with Guinea-pig Ureter.

The ureter suspended in Ringer-Locke solution, in the absence of internal distension or added drugs, was completely quiescent. No tonus was ever observed, and only very occasional "spontaneous" contractions, except towards the end of a long experiment and sometimes in cannulated ureters; in the latter case the stimulating agent was probably the ligature tied over the cannula.

Change in environmental conditions evoked only one type of response, a brief contraction or a series of such contractions. The strip of ureter shortened by 10 to 15 p.c., the contraction being over within 2 or 3 seconds. No persistent tonus was produced by any treatment. The ureter always relaxed completely between twitches, unless the rhythm was very rapid when relaxation was only partial. There was never summation of contractions or a sustained contraction. No grading of height of contraction was observed, except a slight increase during the first few twitches of a series and a decrease when high concentrations of toxic substances were used, that is, the strip of ureter as a whole showed all-or-none behaviour.

The most notable effects were produced by increasing the potassium content of the Ringer-Locke solution and by increasing the hydrostatic pressure within the ureter.

Effect of Potassium.

The concentration of K ion in the Ringer-Locke solution was 22 mg. per 100 ml. An increase in this concentration was followed by a very constant response. This was a regular series of contractions whose frequency varied with the concentration of K. The threshold for the K effect was usually between 2 and 3 times normal concentration. The lowest threshold observed was K × 1·5. In one experiment the following frequencies of contraction were seen (see Fig. 1), K × 1·8, nil. K × 2·6, 1·8 per minute, K × 4·2, 4·0 per minute, K × 5·8, 6·0 per minute. High concentrations, for example K × 15, produced small contractions at a rate of about 16 per minute stopping within half a minute. With moderate concentrations of K the contractions continued for long periods; in one experiment K × 5 caused contractions at a rate of 2·5 per minute; 110 minutes later the ureter was still contracting regularly at 1·5 per minute.

On replacing the potassium-rich solution with normal Ringer-Locke the contractions ceased at once.

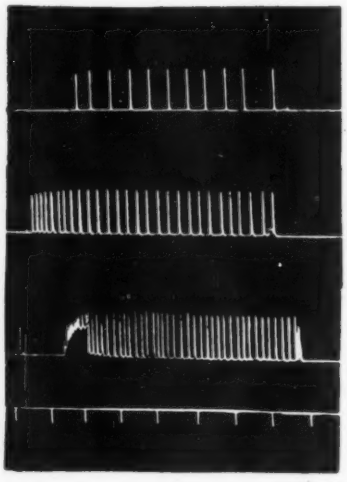


Fig. 1. Contractions of guinea-pig ureter exposed to excess potassium. From above downwards K × 2·6 times normal, K × 4·2, K × 6·8, time scale in minutes.

Barium chloride in concentrations of about 1 in 7,500 had the same effect as excess potassium. In no concentration did it produce a sustained contraction.

Effect of Calcium.

Raising the calcium content of the Ringer-Locke solution never stimulated the ureter, neither did the change from a Ca excess of up to 15-fold back to normal Ringer-Locke. Diminution of Ca content below the normal figure did not stimulate the ureter. Excess Ca slowed or completely stopped the contractions produced by any agent; it neutralized the effect of excess potassium when in roughly equivalent proportion. A constant effect of small increases in Ca concentration, up to about two-fold, was to increase the size of the contractions produced by other agents.

When the ureter was exposed to excess potassium in Ringer-Locke containing less than half the normal concentration of Ca the response ceased after two or three contractions. However, restoration of the normal level of Ca after up to 5 minutes then caused the appearance of repeated contractions at about the rate that would have resulted from the same excess of potassium added to normal Ringer-Locke. That is, even when the response was prevented by deficiency of calcium, the ureter did not become accommodated to excess potassium.

Effect of Distension.

The cannulated ureter, when the pressure inside equalled that outside, was usually completely quiescent. Raising the internal pressure produced a series of contractions, not so regular as those produced by excess potassium but often equally persistent. In one experiment a pressure of 5 cm. of Ringer-Locke induced contractions at a frequency of about 1 per minute which persisted for 60 minutes. The threshold pressure for the production of contractions was about 2 cm. At pressures over 15 cm. the contractions became very small or disappeared. Within this range the frequency of contraction tended to vary with the pressure, though the grading was not nearly so well-marked as that obtained with different concentrations of potassium. The highest rate induced by distension was 2·5 per minute at 5 cm. pressure.

Curare (''Intocostrin'', Squibb) in concentrations up to 1 in 7,500 had no effect on these contractions. Cocaine hydrochloride, 1 in 15,000 decreased the size of the contractions and slightly increased their rate; 1 in 7,500 reduced the contractions to a very small size. Immediately the cocaine was removed the contractions were restored to their previous size. The effect of cocaine on the contractions induced by distension was exactly similar to the effects produced by the same concentrations of cocaine on the contractions induced by barium chloride.

Little evidence of synergism between the effects of potassium and distension was obtained. In some experiments the frequency of contractions produced by excess potassium and distension together was greater than that produced by either agent separately, in other experiments distension did not alter the frequency of contractions produced by excess potassium. Similarly, when the concentration of potassium was reduced below normal a given degree of distension sometimes gave a slower rate of contraction, but in other experiments the frequency did not vary when the potassium concentration was reduced even to one-fifth of its normal value. However, distension and excess potassiom acting together regularly produced contractions of larger size than those produced by either agent separately.

Effect of Acetylcholine.

Acetylcholine had only a slight and variable effect on the ureter. Some ureters showed no response to concentrations of up to 1 in 1,500, others showed an inconstant response, consisting typically of 3 to 5 contractions within 2 minutes, to concentrations of not less than

1 in 75,000. Owing to the inconstancy of the response to acetylcholine it was difficult to determine the influence of atropine and curare. In two experiments the effect of acetylcholine was not altered by atropine, 1 in 75,000, and in two experiments it was not altered by curare, 1 in 7,500. Eserine salicylate, 1 in 1·5 million, slightly increased the number of twitches caused by adding acetylcholine to the bath.

Effect of Adrenaline.

Adrenaline in concentrations up to 1 in 75,000 had no effect on the ureter in four experiments. In four other experiments it inconstantly produced a brief series of contractions. Thus the finding of Burgi (1945) that adrenaline induced rhythmic contractions in the guinea-pig ureter does not seem to hold invariably.

Effect of Nicotine.

Nicotine tartrate in concentrations of up to 1 in 1,500 had no effect on the quiescent ureter. It had, however, in four experiments out of five, an inhibitory effect on the contractions produced by potassium. These contractions were temporarily slowed, and sometimes stopped, by nicotine, 1 in 7,500. A peculiar feature of this action of nicotine was that in three of the experiments it was only apparent on the first two or three applications, thereafter nicotine had no effect on the potassium contractions. Nicotine had a slighter and more evanescent inhibitory action on the contractions induced by distension; it reduced the frequency of the contractions for about two minutes only, the contractions then proceeded as before.

Effect of Histamine.

Histamine constantly stimulated the ureter. The threshold concentration varied between 1 in 375,000 and 1 in 1·5 million. The contractions induced by histamine were less regular than those induced by potassium, tending to occur in groups with pauses between, and the effect was not so prolonged, disappearing within a few minutes.

Experiments with Rabbit and Cat Ureters.

In three experiments the behaviour of rabbit's ureter was of the same nature as that of guinea-pig's ureter, except that the individual contractions were more prolonged, especially when the rhythm was slow, lasting up to 10 seconds. The reactions to excess potassium, distension, acetylcholine, adrenaline, and nicotine were the same. There was a similar absence of tonus or spontaneous contraction.

In one experiment on the cat's ureter excess potassium and distension caused rhythmic contractions, though these were not so regular nor so easily elicited as in the case of the guinea-pig. The individual contractions were again much more prolonged than in the guinea-pig ureter.

DISCUSSION.

The most striking feature of ureteric muscle is its similarity to cardiac muscle. Both have the property, shared by non-adapting receptor cells, of prolonged rhythmic excitation in response to a steady environmental state. Other common features of cardiac and ureteric muscle are the relative rapidity of contraction and relaxation, the all-or-none behaviour of the tissue as a whole,

the absence of tonus and summation of contractions, and (Bozler, 1938) the long refractory period.

That the rhythmicity of ureter, like that of heart, is myogenic is suggested by the effect of cocaine, which abolishes rhythmicity due to distension in the same concentration and at the same rate as it abolishes rhythmicity due to barium. The slight and transitory effect of nicotine in high concentration on the contractions induced by distension is further evidence that no nervous mechanism is involved. The significance of the more marked, but still temporary, inhibitory action of nicotine on contractions induced by potassium remains obscure.

The relative insensitivity of the ureter to acetylcholine and adrenaline raises the question whether there can be any control of its movements by the autonomic nervous system in the animals studied. Presumably the natural stimulus of the ureter to activity is distension with urine. In view of the regularity and constancy of the effect of potassium it is tempting to suppose that distension causes contractions by lowering the threshold of the ureteric muscle to potassium, so that it responds to the normal concentration of potassium present in plasma or Ringer-Locke solution in the same way that the non-distended ureter responds to excess potassium. The existence of such a mechanism would be supported by finding a synergism between the effects of changes in potassium concentration and distension. However, no satisfactory evidence for this synergism was obtained.

SUMMARY.

The isolated ureter of the guinea-pig, rabbit, and cat showed only one type of response to stimulation, brief all-or-none contractions rhythmically repeated.

A long series of rhythmic contractions was induced by exposure of the ureter to a raised concentration of potassium ion, and by internal distension of the ureter.

The ureter was relatively insensitive to acetylcholine and adrenaline.

Some similarities between ureteric and cardiac muscle are pointed out.

REFERENCES.

Bozler, E. (1938): Amer. J. Physiol., 122, p. 614.
Burgi, S. (1945): Helv. physiol. Acta, 3, p. 1.
Gruber, C. M. (1928): J. Urol., 20, p. 27.
Gruber, C. M. (1930): J. Pharm. exper. Ther., 39, p. 449.
Stoicesco, S. (1929): Compt. rend. Soc. Biol., 101, p. 436.